THE USE OF MICROTOX® FOR THE MONITORING OF CRUDE OIL CONTAMINATED SOIL TOXICITY DURING REMEDIATION

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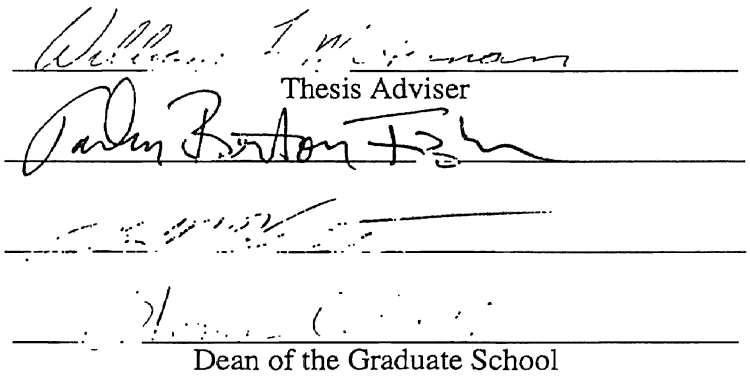

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PREFACE

To evaluate the use of Microtox® toxicity measurement for monitoring crude oil contaminated soil remediation, two of three outdoor test plots containing uncontaminated loam soil were contaminated with 21 gallons of Michigan Silurian crude oil. One crude oil contaminated test plot was amended with fertilizer (NPK), tilled and moisture content adjusted to 80% of container capacity to encourage the growth of hydrocarbon oxidizing microorganisms. The other contaminated test plot was not amended. Evolution of crude oil residues and soil toxicity were monitored in samples taken at random locations from all test plots eight times during the 190-day study. Total petroleum hydrocarbons (TPH), benzene, toluene, ethylbenzene, and xylenes (BTEX) content of each sample were measured using EPA specified methods by a commercial laboratory. Effective concentrations (EC50, EC20, EC10 and EC1) of each sample's water soluble fraction (WSF) were determined by Microtox® basic protocol. TPH values decreased more rapidly in the moisture and nutrient amended plot than in the unamended plot. Microtox® effective concentrations of WSF displayed a more rapid decrease in toxicity in the amended plot. It was determined that no single Microtox® effective concentration metric (EC50, EC20, EC10 or EC1) could describe toxicity variations over the range of toxicity generated in this study. The percent of Microtox® reagent light inhibition produced at 20% WSF provided a consistent means for comparing toxicity changes during the study and showed a good correlation with TPH.

Respirometry tests conducted with samples collected from the test plots at day 140 demonstrated that nutrient and water amended soil had the greatest oxygen demand. This oxygen consumption was presumed to be the result of oxidation of petroleum hydrocarbons by microorganisms.

Qualitative analyses of gas chromatogram patterns showed that TPH in the nutrient and water amended test plot had experienced alterations consistent with extensive biodegradation. Such extensive degradation was not observed in gas chromatograms of samples from the unamended test plot. Degradation of hydrocarbons present in the nutrient and water amended plot had begun extensive degradation in the first 14 days of the experiment.

Microtox® Large Sample Solid Phase (LSPT) and No Observed Effects Concentration (NOEC) protocol tests were performed on samples collected at day 190. Both protocols provide excellent means for distinguishing uncontaminated from contaminated soil. However, both protocols proved to be labor and equipment intensive. Because it is conceptually and operationally simpler to perform, the LSPT was concluded to be more valuable as a Phase II site characterization tool than the NOEC protocol.

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CHAPTER I

INTRODUCTION

Today petroleum producing, petroleum refining and petroleum distribution companies are faced with the challenge of finding cost-effective methods for treating soils contaminated by complex hydrocarbon mixtures (crude oil, gas condensate, diesel fuel, etc.). Whatever method is chosen must reduce the risk to humans and the environment. One of the oldest and most promising methods of achieving the goal of risk reduction is to encourage the growth of a soil's indigenous hydrocarbon oxidizing microorganisms through pH control, moisture control, nutrient addition and aeration. This process is called bioremediation.

Bioremediation is a managed treatment process that uses microorganisms to degrade and transform organic chemicals in contaminated soils, aquifers, sludges and residues (Dasappa and Loehr, 1991). The goal of bioremediation is to degrade and transform chemical contaminants to harmless end products that do not threaten man or the environment. In the ideal case, bioremediation would convert hydrocarbon contamination to biomass, carbon dioxide, inorganic compounds and water. However, when a complex mixture of hydrocarbons contacts soil, it undergoes transformations into new chemicals through biological and chemical reactions that may not result in detoxification and immobilization (Dasappa and Loehr, 1991). The production of daughter compounds can present a greater or equal risk than the original contaminants. The chemical monitoring of all potential toxicants during a remediation is very costly and impractical. The laboratory measurement of reduction in the organic contaminants initially present does not assure that transformation products more toxic than the precursors have not been formed. The use of an effects-based

acute toxicity test as a bioassay to monitor soil toxicity can provide a relatively cheap and rapid method to determine if the goal of environmental cleanup and protection is being met.

It is the goal of this research to evaluate the use of the Microtox® acute toxicity test to compare toxicity evolution in an untreated crude oil contaminated soil to that of a crude oil contaminated soil undergoing a managed treatment process which stimulates the growth of indigenous hydrocarbon oxidizing microorganisms. Michigan Silurian Reef crude oil was applied to two outdoor soil test plots. In one test plot no attempt was made to encourage the growth of indigenous soil microorganisms. In one test plot the soil moisture was adjusted to 80% of container capacity, NPK nutrients added and soil aerated by tilling; the goal being to promote the growth of indigenous hydrocarbon oxidizing microorganisms. A third test plot of uncontaminated test soil was used as a control. Changes in Total Petroleum Hydrocarbons (TPH), BTEX (Benzene, Toluene, Ethyl Benzene and Xylenes) content and the accompanying changes in toxicity were measured in the three test plots for 190 days.

Cleanup standards for contaminated soils are established on a site-specific basis by regulatory agencies. Such site-specific standards should be the result of negotiations between the responsible parities and regulatory agencies. Decisions made should be based on data from both chemical and toxicity measurements made on pilot studies.

State of Michigan regulators have suggested that moisture amendment and tilling of soil contaminated by Michigan Silurian Reef crude oil will increase the risk of contamination at depth. This study will provide data to compare the no action scenario to the managed stimulation of indigenous hydrocarbon oxidizing microorganisms.

CHAPTER II

LITERATURE REVIEW

Biotoxicity Testing

A biological system used for toxicity measurement must have one or more easily measured physiological parameters indicative of its state of health (Bulich, 1979). It is necessary that some simple, rapid and sensitive method for measuring the chosen physiological parameter be available. For these reasons, toxicity tests have generally been limited to a few easily measured effects, such as mortality, growth and reproduction (EPA, 1985).

Acute Toxicity

The objective of an acute toxicity test is to determine the concentration of a contaminant that produces a deleterious effect on a group of test organisms during a short-term exposure under controlled conditions. Acute toxicity tests are effects-based tests that measure the magnitude of response elicited by selected organisms as they react to a chemical at various concentrations.

The easiest of physiological parameters to observe is mortality. The acute mortality test results are expressed as the concentration of toxicant that is lethal to the test organisms over short exposure periods (Munkittrick and Power, 1991). The concentration of toxicant estimated to cause mortality in 50% of the test population is termed LC50. A chemical with a low LC50 concentration is more toxic than a chemical with a higher LC50 concentration. Besides mortality tests, other acute bioeffects-based tests measure the relative effect of various toxicant fractions. Examples of three effects based tests are:

1. The algae (*Selenastrum Capricornutum*) growth test measures changes in cell density, biomass, chlorophyll content, or adsorbance of a *Selenastrum* population exposed to different concentrations of toxicant for 96 hours (EPA, 1985).

2. The Microtox® System used in this study measures the reduction of light output from a luminescent marine bacterium (*Photobacterium phosphoreum*) population challenged by different toxicant concentrations (Bulich, 1979).

3. The Mitochondria RET test measures the production of NAD^+ from the mitochondria of a bovine heart as it is exposed to various concentrations of toxicant. The reduction of NAD^+ is defined as the toxic response (Arctander *et al.*, 1992).

In all the above examples, the EC50 is the toxicant concentration that reduces the measured activity by one half. The LC50 and EC50 concentrations provide a method for establishing relative toxicity of pollutants. This readily understood toxicity measurement has legal validity and is an EPA accepted measure of toxicity. Permits which authorize discharge under the National Pollutant Discharge Elimination System (NPDES) regulate the minimum LC50 allowed (USEPA, 1993).

Bioassay

As stated previously, acute toxicity tests provide a method of measuring the relative concentration of pollutants. Rand and Petrocilli (1985) stated that the acute toxicity test has been erroneously termed a bioassay. They define the bioassay as a test performed to determine the strength of a chemical based on the response elicited by the test organisms compared to the response elicited by the same organism by a standard preparation of the chemical. Bioassays are frequently used in the pharmaceutical industry to evaluate the potency of vitamins and drugs (Rand and Petrocelli, 1985). This study uses the Microtox® acute toxicity test response as a bioassay to track and compare changes in toxicity of a crude

oil contaminated test soil undergoing different remediation scenarios to the toxicity of the uncontaminated test soil.

Chronic Toxicity

The LC50 and EC50 concentrations do not provide an accurate estimate of the toxicant concentration that will cause no observable effects (NOEC) (EPA, 1985). The EPA defines NOEC as the highest concentration of a toxicant to which organisms are exposed in a full life cycle or partial life cycle test, that causes no observable adverse effects on the test organisms (EPA 1989). Chronic toxicity tests that expose populations of test organisms continuously to a chemical for a time sufficient for the controls to grow, develop, become sexually mature, and produce offspring result in NOEC values (Petrocelli, 1985).

Microtox®

Bulich (1979) reported a new, simplified toxicity test that uses luminescent marine bacteria (*Photobacterium phosphoreum)* for determining toxicity of aqueous solutions. Bulich and Isenberg (1980) introduced the Microtox® System as a new technology in the field of aquatic toxicity. The Microtox® System is an instrument-based acute toxicity test that uses the reduction in light output of luminescent bacteria when exposed to toxic substances at 15°C ± 0.5°C. The light producing mechanism in the Microtox® bacteria is the result of oxidation of reduced flavin mononucleotide and a long-chain aldehyde catalyzed by the enzyme luciferase (McGuinness and Barisas, 1991). Microtox® bacteria light inhibition is a measurement of toxicant effects on metabolic pathways linked to the bacterial luciferase system.

Microtox® Bioassay Compared to Other Acute Toxicity Tests

Munkittrick and Power (1991) present a comprehensive review of data correlating the Microtox® acute toxicity test and other acute lethality tests. They deal specifically with

rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*) and *Daphnia*. Microtox®sensitivity when compared to *Daphnia*, rainbow trout and fathead minnow toxicity tests was found to be a function of chemical groups, sediment type, and extraction techniques. For a list of specific relative sensitivities, the reader should refer to Munkittrick and Power (1991). The major conclusions from this paper are summarized as follows:

1. Microtox® appears to be as sensitive as other acute lethality tests to most pure organic compounds. Microtox® is generally more sensitive to complex organic compounds such as multichlorinated benzenes, phenols, and ethanols, but was less sensitive to cyanide, chloroform, or phenol.

2. Microtox® is not as sensitive to inorganics as *Daphnia*, rainbow trout and fathead minnow except for mercury, arsenic, and cobalt.

3. Microtox® has been used with favorable results for monitoring municipal wastes.

4. Microtox® has been useful for monitoring relative changes in toxicity associated with petroleum wastes.

5. As the complexity of industrial effluents increased, the sensitivity of Microtox® increased, and the variability decreased. Microtox® may be useful for screening relative toxicity of highly toxic complex effluents.

6. Microtox® is less sensitive than other acute lethality tests to insecticides, herbicides, pharmaceutical and textile effluents, and highly lipophilic contaminants.

7. Sensitivity of Microtox® varies with contaminant extraction techniques.

8. The Microtox® assay appears to be the best available choice for rapid toxicity assessment of diverse environmental samples.

Since its introduction in 1980, Microtox® has been compared with many accepted acute toxicity tests. Athey *et al.* (1989) compared Alage (*Selenastrum capricornutum*),

Daphnia, lettuce root (*Lactuca sativa*) elongation, earthworm (*Eisenia foetida*) test and Microtox®. They determined that *Daphnia* and Microtox® were most sensitive to toxicity caused by creosote-contaminated water draining from a wood treatment site.

The Norwegian Oil Industry Association (Vic *et al.*, 1992) performed tests to determine toxicity of offshore drilling chemicals. The tests concluded that when compared to the *Artemia* lethality test, Mitochondria RET test, Acrtia lethality test and Alage (*Selenastrum capricornutum*) growth inhibition test, Microtox® was the most sensitive screening test. The test proved to be sensitive to biocides, corrosion inhibitors, and emulsion breakers.

Microtox® Measurement of Acute Toxicity

The use of the Microtox® acute toxicity test has several advantages over what can be termed conventional acute toxicity tests which use animals.

These advantages are as follows:

1. Utilizes a statistically significant population of individuals (1×10^6).
2. Relatively inexpensive when compared to other acute toxicity tests (less than $20 per sample, not including sample preparation).
3. Rapid (15 to 30 minutes, not including sample preparation).
4. Small sample required (25 ml).
5. Lyophilized bacteria are reconstituted at time of use and do not require the skills or facilities for breeding and rearing.

Soil Extract Toxicity

The Microtox® acute toxicity test was introduced (Bulich, 1979) as a simple, rapid method of monitoring the toxicity of "aquatic" samples. In order to apply the Microtox®

Basic protocols to measure the relative toxicity of a soil, sediment or sludge, it is necessary to acquire a liquid extract. Soil treatability studies (Matthews and Hastings, 1987) measured toxicity in the water soluble fraction (WSF) of organic wastes in soils. The water-soluble fractions of organics pose the greatest threat to man and the environment because the primary pathway of contaminant release is leaching to groundwater (Donnelly *et al.*, 1991).

Soil Contact Toxicity

The Microtox® acute toxicity test cannot be conducted when the bacteria are in contact with sediment, because particles absorb the light given off by the bacteria. For this reason, Microtox® measures toxicity of solvent extracts from the sediments. Brouwer *et al.* (1990) discussed a technique that contacted a small quantity of Photobacterium to a test sediment in liquid suspension. After 15 minutes of incubation in contact with sediment, the suspension was centrifuged and the light output of 1 ml of supernatant measured. Brouwer *et al.* (1990) stated that the greatest advantage of sediment contact is that the toxicity of the entire sediment is measured. This direct contact method often indicates a higher toxicity value than does an extract of a contaminated sample. Greene *et al.* (1992) theorized that the direct contact makes toxicants adsorbed on particles more bioavailable by some mechanism yet to be explored. Potential sources of error in the direct contact method were enumerated by Greene *et al.* (1992):

1. Bacteria may be lost from solution by attachment to solids.
2. Representative small samples of sediment are difficult to obtain.
3. Residual fine particles in solution interfere optically with light measurement.

To reduce the impact of these sources of error the Microbics Corp. (1992) published a sediment contact protocol referred to as the "large sample procedure" (LSPT). The LSPT protocol uses a large initial sample (7 grams) in order to obtain a representative sample.

Photobacterium are incubated for twenty minutes in contact with thirteen 1:2 serial dilutions of a sediment and Microtox® diluent suspension. Solids are settled and filtered from the suspensions and light values measured. The Microbics (1992) reference file method of data reductions allows that all toxic responses can be calculated relative to another file. This "reference file" is the sample of the same sediment which produces the smallest toxic response at the largest concentration. This "reference file" technique has the purpose of removing the effects of soil chemistry and soil particle size on the measured toxicity. See Appendix E for details of Microtox® LSPT protocol.

Microtox® Measurement of NOEC

As discussed previously, the No Observed Effects Concentration (NOEC) has been defined using full life cycle tests. As shorter tests were developed, it became common practice to apply the same terminology to endpoints of these tests (EPA, 1989). As used today, the NOEC can be defined as the highest concentration of toxicant in which the values for observed parameters are not statistically significantly different from the controls. This definition of NOEC requires two assumptions. First, it must be assumed that if an effect is not statistically observable, it is not significant from a biological standpoint. Secondly, it is assumed that there exists a true threshold or concentration below which there is no adverse effect and above which there is an adverse effect (EPA, 1989). The Microbics Corporation (Microbics, 1992) developed an NOEC test protocol and data reduction software which utilizes Dunnett's procedure to determine NOEC. Dunnett's procedure uses analysis of variance (ANOVA) to compare the mean light inhibition of bacteria at each toxicant concentration with the control inhibition mean to determine at which concentration there exists no observable difference in inhibition (USEPA, 1989). The EPA recommends that the Dunnett's hypothesis be used for the Microtox® experimental design (Microbics, 1992).

Field Evaluation of *In Situ* Hydrocarbon Biodegradation

The field evaluation of *in situ* biodegradation of hydrocarbons is more difficult than in a laboratory study (Atlas, 1991). One to ten percent of bacteria observed in uncontaminated soils are capable of using hydrocarbons as the sole source of carbon and energy. The presence of hydrocarbons in the environment frequently causes the selective *in situ* enrichment of these hydrocarbon oxidizing microorganisms (Rosenberg and Gutnick 1981). Rosenberg and Gutnick (1981) have stated that the determination of the concentration of hydrocarbon degrading bacteria is one of the methods commonly used for identifying oil pollution in the environment. However, the enumeration of hydrocarbon degrading organism colonies on agar-based hydrocarbon media must be approached with caution because tests have often shown that less than 30 percent of the organisms that form colonies on oil agar are capable of metabolizing hydrocarbons (Atlas, 1991). Field evaluations must rely on enumeration of microorganisms and the analysis of residual hydrocarbons and reduction of toxicity. It is important in any field bioremediation to have an untreated reference site for comparison (Atlas, 1991). This need for a reference site as control is often overshadowed by the desire to clean up all pollutants in the most timely manner.

Enhancement of *In Situ* Hydrocarbon Biodegradation

It is known that soils have a diverse population of microorganisms with the capability of oxidizing crude oil. The rate of crude oil oxidation is controlled by the concentration and composition of hydrocarbons, nutrient status, aeration, moisture, pH and temperature (Deuel, 1991). Hydrocarbons are not biodegraded under anaerobic conditions at rates that can be used to remediate hydrocarbon contaminated sites; therefore, biodegradation at rapid rates requires a sufficient supply of molecular oxygen (Atlas, 1991).

Wang and Bartha (1990) studied fuel spill bioremediation and determined that the application of fertilizer plus tilling strongly decreased fuel persistence, toxicity and

increased microbial activity when compared to contaminated but untreated soils. Deuel (1991) recommended that Nitrogen (N) be added to the contaminated soil using ammonium sulfate or urea at rates to provide an oil and gas to N ratio of 150:1. Phosphorous (P) and potassium (K) content added should provide a N:P:K ratio of 4:1:1.

Respirometry

The measurement of oxygen consumption is used as a means of determining biodegradability. Respirometry is one method for measuring oxygen consumption; it uses a batch reactor incubated at constant temperature containing substrate and biomass in contact with gaseous source of oxygen. Oxygen uptake by microorganisms over a period of time is measured by change in volume or pressure of the gas phase within the reactor. An alkali is included in the reactor to absorb carbon dioxide produced (Dessai *et al.,* 1990). Respirometry may be used for determination of biochemical oxygen demand (BOD) and biodegradation kinetics. Oxygen demand of identical hydrocarbon-contaminated soil samples inoculated with microorganisms from different sources should provide a relative population measure of hydrocarbon-oxidizing microorganisms in each innoculum.

Gas Chromatograph Of Biodegraded Hydrocarbons

Microorganisms must break bonds between carbon atoms before they can consume hydrocarbons. For this reason, hydrocarbons differ in their susceptibility to microorganism attack. Generally, hydrocarbons have been ranked in the following order of decreasing susceptibility: *n*-alkanes, branched alkanes, low molecular aromatics and cyclic alkanes (Leahy and Cowell, 1990). This difference in susceptibility of hydrocarbons to microorganism breakdown causes an alteration in the gas chromatographs obtained from crude oil as degradation proceeds. Figure 1 shows crude oil degradation effects on the gas chromatographs. The undegraded crude oil gas chromatograph shows a rich spectra of *n*-alkanes and branched alkanes. The gas chromatograph of the moderately degraded crude oil shows that

the *n*-alkanes have been removed. In the severely degraded crude oil, the branched alkanes are gone and much of the chromatograph consists of a large "hump" referred to as the Unresolved Complex Mixture or UCM (Gough, 1989). The UCM appears to consist of compounds which are relatively inert to microbial degradation, although the exact nature of these compounds is unclear (Gough, 1989).

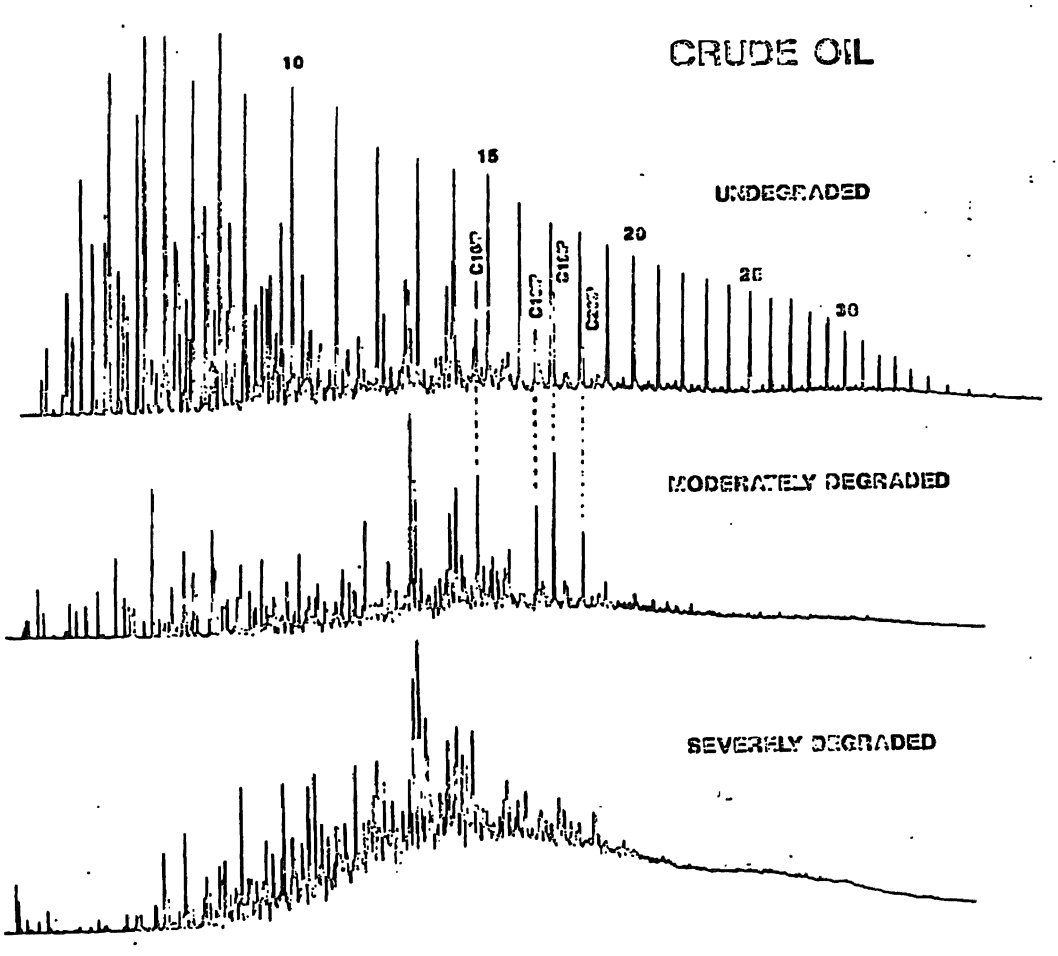


Figure 1. Effects of Crude Oil Degradation on Gas Chromatograph Pattern (Amoco Corp., 1989)

CHAPTER III

EXPERIMENTAL MATERIALS AND METHODS

Test Site Location and Test Plot Construction

The *in situ* crude oil remediation experiment was conducted in three soil test plots located at the Amoco Production Company environmental research site located in the West 21.49 acres of government Lot 3 in Section 31, Township 21, North Range 15, East Rogers County, Oklahoma. Each soil container is constructed of reinforced concrete with inside dimensions of 9.1 feet x 9.1 feet x 3 feet deep. Each test plot contains 9.2 cubic yards of soil.

Test Soil

The soil used to fill the three test plots was collected in Lot 3, NW Section 6, Township 17 North, Range 15, East Tulsa County, Oklahoma. This soil is representative of the Okay Series (Cole, 1977). The Okay Soil Series consists of deep, moderately permeable, level to gently sloping upland soils that have formed in loamy material under native grasses. Values of EC = 1 mmhos/cm, SAR < 1.0 and ESP < 1.0 indicate that test soil has not been contaminated by Sodium. Values of TPH IR = 17.5 ppm and TPH GC indicate that soil has not been contaminated by oil (Deuel, 1991).

Test Soil Characteristics

The test soil placed in the test plots was allowed to settled for three months. At the end of three months, vegetation was removed and duplicate samples consisting of mixtures of soil from the three test plots were collected. These samples were tested to establish phys-

ical and chemical characteristics of the soil. The container capacity of the soil was determined by a method modified from Cassel and Nielsen (1962). One hundred grams of test soil were placed in a 110 ml polypropylene container that had 30 approximately 1/32 inch (.79 mm) holes pierced in its base. Soil was thoroughly wetted and water allowed to drain. Container was covered to prevent evaporation and soil was allowed to drain for six hours. At the end of the drainage period, a small sample, approximately 10 grams, was tested for moisture content using a Denver Instruments Soil Moisture balance. The average of four such measurements was determined to be the container capacity of the test soil. Container capacity determined by the above method was treated as a surrogate of field capacity and was used to approximate water augmentation required to bring soil to 80% of field capacity as called for in Test Plot 3.

Soil Analytical Services Inc., College Station, Texas, conducted all chemical tests on soil samples. The tests consisted of the following: Saturated Paste (SP) Moisture, pH, SP Electrical Conductivity, Soluble Cations, Sodium Adsorption Ratio (SAR), Cation Exchange Capacity (CEC), Exchangeable Cations, Exchangable Sodium Percentage (ESP), Base Saturation, Total Organic Carbon (TOC), Total Petroleum Hydrocarbons (TPH) by Infrared Spectrometry, and Total Petroleum Hydrocarbons by Gas Chromatography. Infrared measurements were based on EPA 418.1 and GC analyses were conducted according to standard method 8015. EPA method 8020 was used to measure BTEX content of crude oil contaminated soil. Appendix A discusses these measurements in more detail. The Texas Railroad Commission has authorized Soil Analytical Services Incorporated to conduct EPA soil testing (Barker, 1992).

Michigan Silurian Reef Crude

The Silurian Reef Crude oil used in this remediation experiment came from the Amoco Production Company's Rabourn lease, Michigan. Raterman *et al.* (1993) con-

ducted Gas Chromatography type analysis of this crude oil. The test sample taken from the oil-water separator was sealed at time of collection to retain volatiles.

Contamination of Soil Test Plots

Test plot No. 1 was not contaminated and served as a control for the duration of the experiment. Twenty-one gallons (79,499 cm^3) of Michigan Silurian Reef crude oil from the Amoco Production Company's Rabourn lease, Michigan, were applied to the soil in test plots No. 2 and No. 3 using a hand-pumped garden sprayer. Crude oil was applied as evenly as possible with this application method. Crude oil was tilled into the upper one foot of each plot. Based on a soil weight of 97 pounds per cubic foot (1.5 g/cm^3) and oil density of.78 g/cm^3, the loading rate by weight is approximately equal to 17,000 mg/kg (1.7%). Studies indicate that low level loading rates of 1-13% to the upper one foot of soil pose no problem of leaching to depth (Duel, 1991). Wang (1991) states that lethality to aerobic microorganisms is rare when oil is added to soil, especially when lighter fractions have evaporated.

Postcontamination Test Plot Treatments

Three soil conditions were considered in this study. Test plot No. 1 was not contaminated with crude oil. A waterproof tarpaulin covered test plot No. 1 for the duration of the experiment. After crude oil application, the soil in test plot No. 2 and test plot No. 3 was tilled to a depth of 12 inches (30.5 cm). Test plot No. 2 was covered with a waterproof tarpaulin for the duration of the experiment. Fertilizer was applied to test plot No. 3 consisting of 5.12 pounds (2.32 kg) of 4-1-1 (NPK). The fertilizer was a mixture of 3.6 pounds (1.63 kg) of 45-0-0 as Urea, 0.87 pound (.39 kg) of 0-46-0 as P_2O_5 and 0.65 pound (.29 kg) of 0-0-62 as K_2O. This application provided 1.6 pounds (.73 kg) Nitrogen, 0.4 pound (.18 kg) of Phosphorous and 0.4 pound (.18 kg) Potassium. The NPK ratio is equal to 4:1:1, and the estimated oil to nitrogen ratio is 85:1. This application is consistent with the rec-

ommendations of Deuel (1991), who advises that Nitrogen (N) be added to the receiving soil in the form of ammonium sulfate or urea at rates to provide an N:P:K ratio of 4:1:1. This application provides 1 pound (.45 kg) of Nitrogen per 85 pounds (38.56 kg) of crude oil applied. This is approximately 1.8 times the recommended O&G:N ratio of 150:1 (Deuel,1991). Test plot No. 3 was covered with a waterproof tarpaulin for the duration of the experiment, tilled once each week and water content adjusted to keep moisture content at approximately 80% of container capacity.

Soil Sampling

Before the initial sampling event, each 9.1 feet (2.77 m) x 9.1 feet (2.77 m) test plot was divided into four 4.55 feet (1.385 m) x 4.55 feet (1.385 m) quadrants. The quadrants were created by using a carpenter's chalk line strung tightly between indexing points marked on the test plots' four sides. These index points provided a means for the precise reestablishment of the four quadrants during subsequent sampling events. The common corner of the four quadrants provided an origin from which all sample coordinates were measured. A list of random x,y coordinates between 0 and 54 inches were generated utilizing the SAS Random Number Generator available on the Amoco Production Company Research Center computer system (Fisher, 1992). All sampling locations were selected from a list of coordinates for the quadrant in the test plot being sampled. Care was taken not to sample the same point twice during the duration of the experiment. This was accomplished by plotting all sample coordinates on a map of each test plot. Appendix B is a list of each sample taken and the coordinates of that sample. Soil samples were collected 14, 28, 42, 56, 118, 151 and 190 days after contamination. Samples were collected utilizing a 1-1/8 inch (2.86 cm) x 24 inch (60.96 cm) AMS slotted alloy soil probe with an extension for samples deeper than 24 inches. Care was taken to clean the soil probe after each interval was sampled using available water and wiping the probe dry. Using this type sampling equipment, the probe must pass through shallow contaminated layers, making it impossible

to recover a deep sample that does not have a potential for minimal contamination from the shallow layer. For this reason, only four samples were taken from the 12-24 inch (30.48-60.96 cm) and 24-36 inch (60.96-91.44 cm) intervals during each sampling event.

During each sampling event, four random samples were collected in each quadrant from the 0-12 inch (0-30.48 cm) interval of a test plot. The composite of these four quadrant subsamples was the 0-12 inch (0-30.48 cm) sample for that quadrant. During each sampling event, one sample was collected from the 12-24 inch (30.48-60.96 cm) interval from each quadrant. The composite of these four subsamples was the sample for the 12-24 inch (30.48-60.96 cm) interval of the test plot. The 24-36 inch (60.96-91.44 cm) interval samples were obtained in the same manner as the 12-24 inch (30.48-60.96 cm) interval sample. Subsamples representing a specific quadrant or depth interval were combined and thoroughly mixed to obtain a sample considered representative of that quadrant or depth interval. Each sample was divided into two aliquots which were placed in 8 ounce (236.58 cc) metal cans. The cans were sealed and marked with a sample number indicative of the sample event, test plot, quadrant and depth. All samples were placed in an ice chest with melting ice, returned to the laboratory and stored at 4°C (39.2°F). One of the canned sample pairs was sent by overnight express mail to Soil Analytical Services Incorporated, College Station, Texas, for chemical testing and the other was used for Microtox® toxicity testing.

Relative Abundance of Hydrocarbon Oxidizing Microorganism Population By Respirometry

Experiment Design

In this and any other system undergoing remediation, the question becomes one of whether the observed losses are due to physical loss, chemical transformation or actual biodegradation. A respirometry experiment was designed to demonstrate that a cell mass of

hydrocarbon oxidizing microorganisms was present in the test soil and was exerting the largest effect in the test plot that was losing contaminants at the highest rate. The respirometry experiment measured the oxygen demand of sterilzed test soil samples contaminated with sterilized Michigan Silurian Reef crude inoculated with 10 grams of soil collected on day 140 from each test plot. The oxygen demand of the soil, oil and inoculum mixture is considered representative of the number of acclimated hydrocarbon oxidizing microorganisms present in the inoculum.

Sample Preparation

Eight respirometer bottles, 400 grams of test soil and 1 liter of Evan's nutrient solution (Rosenburg and Gutnick, 1981) were sterilized in a Harvard/LTE Benchtop 90 Multicycle Autoclave. Twenty-five ml of Michigan Silurian crude oil were sterilized by passing through a 0.2 micron Teflon centrifugal filter. A sample containing approximately 40.00 grams, ±0.05 grams of a mixture containing by weight to following: 82% sterilized test soil, 3% sterilized Michigan Silurian Reef crude and 14% Evan's nutrient solution was placed in each respirometer bottle. Two respirometer bottles were inoculated with 10 grams of sterilized soil as control. One of the remaining three pairs of respirometer bottles was inoculated with 10 grams of soil from test plot No. 1. One pair was inoculated with 10 grams of soil from test plot No. 2. The last pair was inoculated with 10 grams of soil from test plot No. 3. All eight respirometer bottles were placed in an N-CON Systems Company Incorporated, 2410 Boston Post Road, Larchmont, NY, 10538, COMPUT-OX SERIES WB 512 differential pressure respirometer. The system consists of temperature controlled water bath into which ten respirometer bottles and their contents can be placed. When connected by hose to the system, each sealed bottle's initial pressure is measured. Respiration of microorganism causes a reduction in pressure which is sensed electronically and oxygen is delivered to the bottle, returning it to initial pressure. This oxygen delivery volume is recorded digitally. The CO_2 generated by the respiration is absorbed by KOH.

The oxygen demand of the eight respirometer experiments was monitored at 25°C for a period of 340 hours. The duration of this experiment was limited by equipment availability.

Microtox®Assay of Soil Toxicity

Extraction of Soil Water Soluble Fraction

The water soluble fraction (WSF) extraction technique used in this study was that of Matthews and Hasting (1987) with minor modification. An aqueous extract of each soil sample was obtained by mixing 100 grams of soil with 400 ml of de-ionized water in a 2,000 ml borosilicate glass tumbling bottle tightly sealed with a Teflon-lined screw cap. Each tumbling bottle was mixed with a rotary tumbler for 22 ± 2 hours at a speed of 30 revolutions per minute. After tumbling, each soil water mixture was allowed to settle for one hour. Liquid from each tumbling bottle was then decanted into a 135 ml flask and stored at 4°C. At the time of testing, supernatant from the 135 ml flask was centrifuged for 30 minutes at 3,600 revolutions per minute. Any suspended material in the centrifuged supernatant was removed by filtering through a 0.45 micron centrifugal filter. Toxicity tests were conducted on samples within six hours of the final extraction step.

Microtox® Acute Toxicity of Water Soluble Fraction

Microtox® acute toxicity (EC50) of the WSF of all contaminated soils considered in this study was determined using the Microtox® basic protocol with one control and four 1:2 Serial Dilutions (Microbics Manual, 1992). Appendix E presents the Microtox® protocols used in this study in detail. A Microbics Corporation Microtox® Model 500 Analyzer was used to measure the response of the Microtox® reagent to soil sample WSF at 5 and 15 minutes. All data collection was done manually and later input into Microbics Corporation MTX7 data reduction software to calculate EC50 values. The Microtox® basic protocol as used in this study measured the light inhibition caused by 45%, 22.5%, 11.25%

and 5.65% dilutions of the WSF compared to a blank. The light inhibition values for each concentration were reduced to gamma (Γ) values by the following equation:

$$\Gamma = \frac{I_o \times R_t}{I_t} - 1 \qquad (1)$$

I_o = light value at time zero before addition of toxicant

I_t = light value at time t after addition of toxicant

R_t = light inhibition correction factor determined from blank

$$R_t = \frac{Blank \text{ light at time t}}{Blank \text{ light at time 0}}$$

at $\Gamma = 1$, when light is inhibited 50%

A graph of Log Gamma vs. Log Concentration was constructed and used to determine effective concentration values.

Microtox® No Observed Effects Concentration (NOEC) of Water Soluble Fraction

Microtox®No Observed Effects Concentration (NOEC) of sample event 9 soil samples WSF was determined using the Microtox®NOEC protocol, which uses one control and five dilutions in quadruplicate (Microbics Manual, 1992). A Microbics Corporation Microtox® Model 500 Analyzer was used to measure the response of the Microtox® reagent to soil sample WSF at 5 and 15 minutes. To obtain concentrations of WSF which would have no observed response (NOEC) required several dilutions. These dilutions were made using the same de-ionized water used for soil extractions. All data collection was done manually and later input into Microbics Corporation MTX7 data reduction software to calculate NOEC values.

Microtox® Solid Phase Soil Toxicity

The Microtox® protocol for large sample solid phase test (LSP) was used to determine solid phase acute toxicity (EC50) of soil samples from sample event 9 (Microbics

Manual 1992). The LSP measures the reaction of Microtox® reagent as it is contacted with thirteen 1:2 serial dilutions of soil and Microtox® Solid Phase Diluent. This test is run in duplicate using two controls. A Microbics Corporation Microtox® Model 500 Analyzer was used to measure the response of the Microtox® reagent to soil and diluent dilutions at 5 minutes. As in other tests, the data from this test were recorded manually and later input into Microbics Corporation MTX7 data reduction software to calculate EC50 values for solid phase tests.

Toxicity of Laboratory Contaminated Test Soil

Five samples of test soil were contaminated with Michigan Silurian Crude in the laboratory at the 1, 2, 3, 4 and 5 percent by weight as outlined in Table I. This contamination was accomplished by weighing air-dried test soil into a glass 225 ml Erlenmeyer flask on a Mettler PM400 scale. Crude Oil was added using a micropipettor until the total weight was 100 grams.

TABLE I

LABORATORY CONTAMINATION OF TEST SOIL SAMPLES WITH MICHIGAN SILURIAN REEF CRUDE OIL

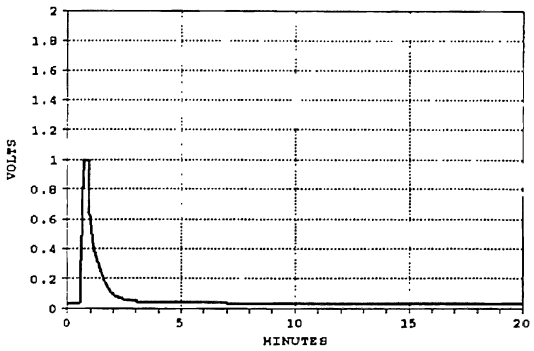
Contamination % by Weight	Weight of Soil	Weight of Crude
BLANK	100 grams	0
1%	99 grams	1 gram
2%	98 grams	2 grams
3%	97 grams	3 grams
4%	96 grams	4 grams
5%	95 grams	5 grams

Flasks containing contaminated soil were placed in 2,000 ml glass tumbling bottles and cushioned with a Styrofoam packing material. Each tumbling bottle was mixed with a rotary tumbler for 48 hours at a speed of 30 revolutions per minute. After tumbling, the WSF was extracted using the technique previously described and the Microtox® acute toxicity test (basic protocol) was conducted on each of the six samples.

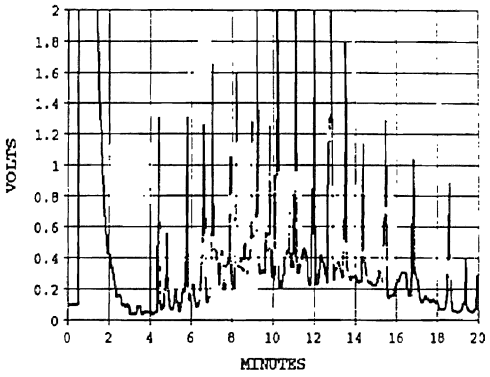
Gas Chromatograph Pattern Recognition

Gas chromatographs obtained in this study were not available in digital format and digitization of paper prints proved to be an unrewarding task due to the poor quality of graphs. Pattern recognition proved to be the most practical way of obtaining any information about the degradation of hydrocarbons in the contaminated test plots.

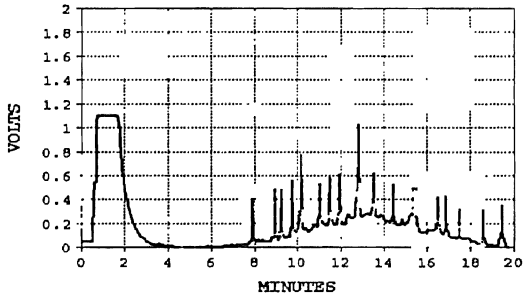
Chromatographs were placed into four pattern recognition classes which are described as follows: Class 1 is the gas chromatograph of the uncontaminated soil that shows no hydrocarbons present; Class 2 is the gas chromatograph identified by a rich spectra of n-alkanes and branched alkanes characteristic of an undegraded crude oil; Class 3 is the chromatograph characterized by a depletion of the Class 2 spectra with the development of an Unresolved Complex Mixture (UCM) "hump" and obvious spike at 12.82 minutes indicative of a biodegraded crude oil; and Class 4 is the unusuable gas chromatograph due to some obvious error. Figure 2 illustrates an example of classes 1-3.



Class 1



Class 2



Class 3

Figure 2. Gas Chromatograph Pattern Recognition Class 1, Class 2 and Class 3

CHAPTER IV

RESULTS AND DISCUSSION

Test Soil Characteristics

Test Soil Selected Physical and Chemical Characteristic

Table II presents the average value of selected chemical and physical characteristics of duplicate samples of test soil which were chosen for measurement before contamination with crude oil. Appendix A presents a brief definition of each characteristic.

TABLE II

SELECTED PHYSICAL AND CHEMICAL CHARACTERISTICS OF TEST SOIL

Moisture, %	9.6	ESP, %	<1.0
SP Moisture	26.4	Base Saturation,%	65.5
pH	7.0		
SP EC, mmhos/cm	1.0	SAR	<1.0
CEC, meq/100g	4.8	TOC, ppm	0.2
NO_3-N, ppm	0.3	TPH-IR, ppm	17.5
PO_4-P, ppm	30.2	TPH-CG, ppm	4.1
EDTA K, ppm	61.7		
Soluble Cations mg/l		Exchangeable Cations meq/100g	
Sodium	0.2	Sodium	<0.1
Calcium	1.9	Calcium	2.1
Magnesium	0.9	Magnesium	1.0

Based on these measurements, it can be concluded that the test soil is a fertile loam soil that has not been contaminated by oil field wastes prior to this study. EC <1.0 mmhos/cm, SAR <1.0 and ESP <1.0 indicate that sodium contamination has not taken place. TPH IR = 17.5 ppm and TPH CG <25 ppm rule out oil contamination.

Type Analysis of Michigan Silurian Reef Crude Oil

Type analysis of the crude oil indicated an average mole weight of 184 with alkane numbers raging from C_4 - C_{53} (Raterman *et al.*, 1993). Table III presents the results of the type analysis of the crude oil.

TABLE III

TYPE ANALYSIS OF SILURIAN REEF CRUDE

(Raterman et al., 1993)

Carbon Number	Mole Percent
C_4-C_{10}	48.98
C_{11}-C_{20}	36.66
C_{21}-C_{30}	10.26
C_{31}-C_{40}	3.05
C_{41}-C_{53}	1.05

This analysis establishes that the Silurian crude oil is a light crude with nearly 50 mole percent having a carbon number of C_4-C_{10}. High volatilization losses can be expected in fresh spills of this crude oil (Raterman *et al.*, 1993).

Toxicity of Michigan Silurian Reef Crude Oil Contaminated Test Soil

The Microtox® Basic Protocol EC50 values of WSF extracts from test soils contaminated in the laboratory are outlined in Table IV. EC50 values are expressed as a percent of WSF. All extractions were made with the same .25 grams of soil to 1 ml of DI water ratio; therefore, percentage of WSF is the most practical unit for comparing effective concentrations. Figure 3 presents these values as a graph of Log Gamma (Γ) vs. Log Concentration of WSF. Table IV and Figure 3 both demonstrate that the addition of Michigan Silurian crude oil increases the test soils WSF Microtox®toxicity. This is observed in the reduction of EC50 values as the concentration of crude in the soil increases.

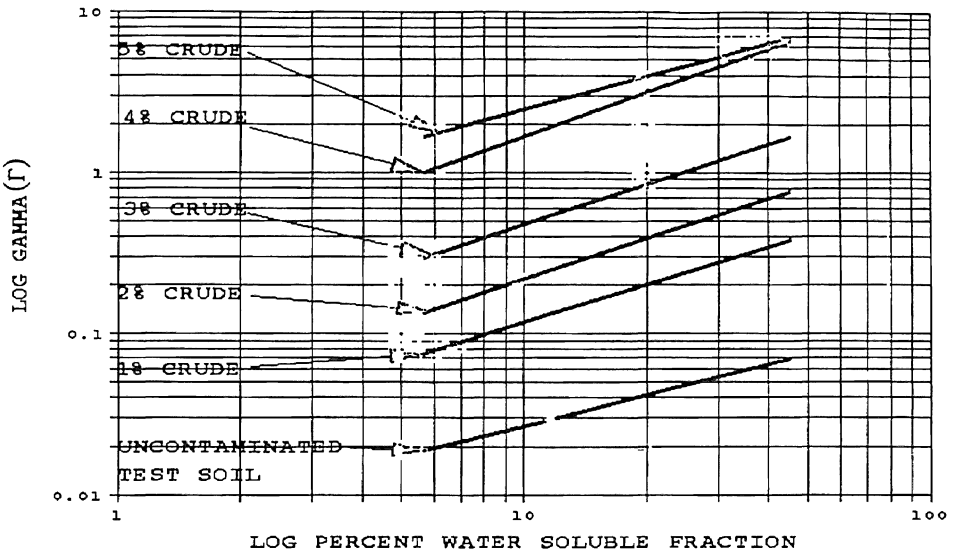


Figure 3. Log Gamma (Γ) vs. Log Percent WSF of Crude Oil Contaminated Test Soil

TABLE IV

MICROTOX® EC50 OF WSF EXTRACTS FROM TEST SOIL CONTAMINATED WITH MICHIGAN SILURIAN CRUDE

PERCENT CONTAMINATION BY WEIGHT	EC50 PERCENT OF WSF EXTRACT	95% CONFIDENCE RANGE
0.0	2074.0	29.0-145950.0
1.0	136.4	13.8-1352.1
2.0	58.8	22.1-156.5
3.0	24.5	18.9-31.6
4.0	5.7	4.5-7.0
5.0	2.5	0.9 -6.7

Relative Abundance of Hydrocarbon Oxidizing Microorganism Population By Respirometry

Figure 4 is a graph of oxygen uptake in mg/kg vs. time (hours). The inoculum from the fertilizer amended test plot No. 3 developed a measurable oxygen demand within 30 hours of experiment initiation. This demand continued for the 344 hour duration of the experiment at an average rate of 35.1 mg/kg per hour. Test plot No. 2 inoculum had a mea-

surable oxygen uptake which was initiated at 93 hours and continued at the average rate of 9.0 mg/kg per hour for the extent of the experiment. Inoculum from uncontaminated Test plot No. 1 had a measurable oxygen demand after 193 hours of 3.8 mg/kg. Test plot No. 1 inoculum had an average oxygen demand of 3.8 mg/kg per hour. Sterile control inoculum and sterile crude and soil mixture did not develop an oxygen demand during the experiment. This respirometry experiment demonstrated that the test soil contained microorganisms capable of utilizing hydrocarbons. The oxygen uptake of Test plot No. 1 inoculum is the evidence for this conclusion. Test plot No. 3 developed a microorganism population which had an earlier onset of oxygen demand and a larger oxygen demand than both Test Plots 1 and 2. It is inferred from this that Test Plot 3 contained microorganisms more efficient in utilization of the crude oil substrate than did Test Plots 1 and 2.

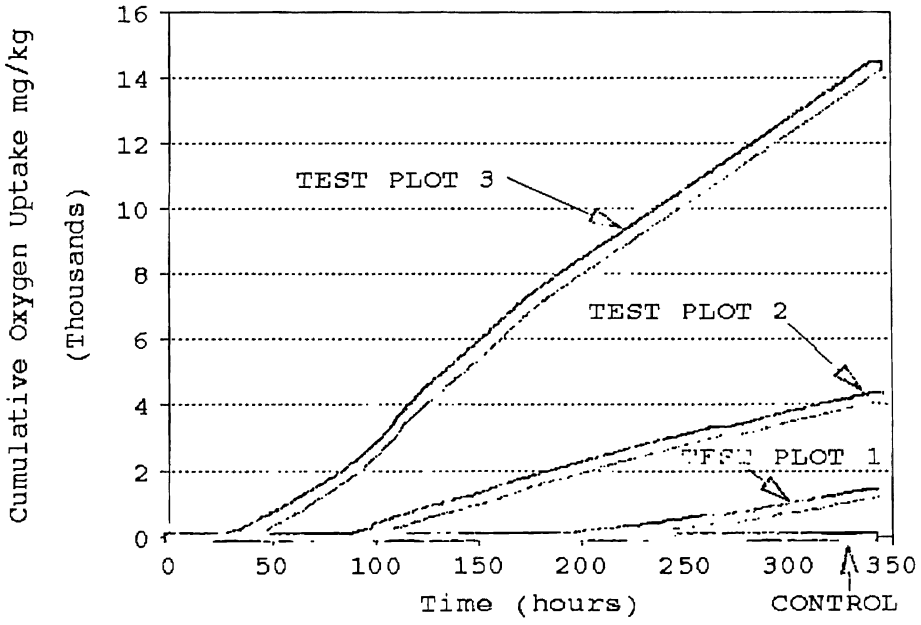


Figure 4. Cumulative Oxygen Demand of Sterile Crude Oil and Soil Mixtures Inoculated with Soil from Test Plots

Gas Chromatograph Pattern Recognition

As discussed above, four gas chromatograph patterns were recognized in the 123 gas chromatographs obtained in this remediation study. Table V summarizes the distribution of the patterns by test plot, depth and sample event. Forty-five of the gas chromatographs were representative of Class 1 which displayed no describable spectra. Thirty-three were from soil samples taken from Test plot No. 1 and 11 were from soil samples taken from Test plot No. 3. Class 2 represented by a rich spectra of alkanes and n-alkanes was found in a total of 45 soil samples. Forty-three of these were from Test plot No. 2 and two from Test plot No. 3. Class 3 was found in a total of 31 chromatographs which were all from Test plot No. 3. Class 3 displays a depleted Class 2 spectra with a well-developed UCM "hump" and is considered representative of biodegraded hydrocarbons. The gas chromatographs of Test plot No. 3 reached this degraded level by the fourteenth day of the experiment. Test plot No. 2 gas chromatographs did not reach the degraded Class 3 pattern for the 190-day experiment.

TABLE V

NUMBER OF GAS CHROMATOGRAPHS PER PATTERN RECOGNITION CLASS FOR TEST PLOTS 1, 2 AND 3

DAY	DEPTH FT	TEST PLOT 1				TEST PLOT 2				TEST PLOT 3			
CLASS		1	2	3	4	1	2	3	4	1	2	3	4
14	1	3			1		4					4	
	2						1			1			
	3						1			1			
28	1						4					4	
	2												
	3												
42	1						4				2	1	1
	2									1			
	3											1	
56	1	4					4					4	
	2	1					1					1	
	3	1					1					1	
88	1	4					4					3	
	2	1					1					1	
	3	1					1					1	
118	1	4					3		1			4	
	2	1					1					1	
	3	1					1					1	
151	1	4					4					4	
	2	1					1			1			
	3	1					1			1			
190	1	4					4			4			
	2	1					1			1			
	3	1					1			1			

Total Petroleum Hydrocarbon Content of Test Plot Soil

The total petroleum hydrocarbon (TPH) content of soil samples in parts per million is presented in Appendix C, Table X. The TPH contents determined by Gas Chromatography (GC) and Infrared Spectrometry (IR) are both presented. Figures 33-35 in Appendix C are TPH IR vs. Depth graphs of each test plot over the three test intervals for each sample event. The minimum detection limit of Gas Chromatography (GC) is 25 ppm TPH and the

minimum detection limit of Infrared Spectrometry (IR) is 1 ppm TPH. The poor quality of gas chromatographs acquired during this study caused their quantitative value to be suspect. For these reasons, only TPH IR values are considered in this discussion. Figure 5 summarizes total petroleum hydrocarbon concentrations as graphs of TPH IR in parts per million versus days for each test plot. The uncontaminated test plot No. 1 had very small levels of TPH over the 190-day history of the study. The maximum TPH value measured was 79.0 ppm and the minimum 7.9 ppm with a median concentration of 24.0 ppm. The 14-day median TPH value of 23,635 ppm in the upper one foot of test plot No. 2 compared to 18,871 ppm in test plot No. 3 and was initially considered a function of soil heterogeneity and unequal crude oil application. Analysis of gas chromatograph patterns, however, indicated that test plot No. 3 had at this early date undergone some degradation, which could explain the differences in TPH concentration. During the period of day 14 until day 88, the TPH content of the upper one foot of test plot No. 2 was reduced 10,420 ppm (44.1%). Over the same period, test plot No. 3 exhibited a TPH reduction of 9,835 ppm (52.1%). On day 118, samples from the upper one foot interval of test plot No. 2 recorded a TPH concentration increase of 24,800 ppm (51.2%) over the day 14 concentration. This sudden increase in TPH concentration was briefly considered to be a laboratory error but was confirmed by low Microtox® effective concentration values. The increase in day 118 TPH concentration is considered to be a function of uneven crude oil application to test plot No. 2. In the upper one foot of test plot No. 3, day 56, TPH concentration reached a median of 7,937 ppm. During the next three sampling events, the TPH concentration varied an average of only ±626 ppm. On day 190, the TPH concentration in the upper one foot of test plot No. 3 reached a minimum value of 2,210 ppm. This minimum value is a reduction of 88.3% in TPH between day 14 until day 190 in the upper one foot interval of test plot No. 3. During the same period, test plot No. 2 had a TPH concentration reduction of only 26.3%. Measurements of TPH concentration in the 1-2 ft interval of test plot No. 2 reached a maximum of 9,471 ppm on day 88. The deeper 2-3 ft interval of the same plot reached a

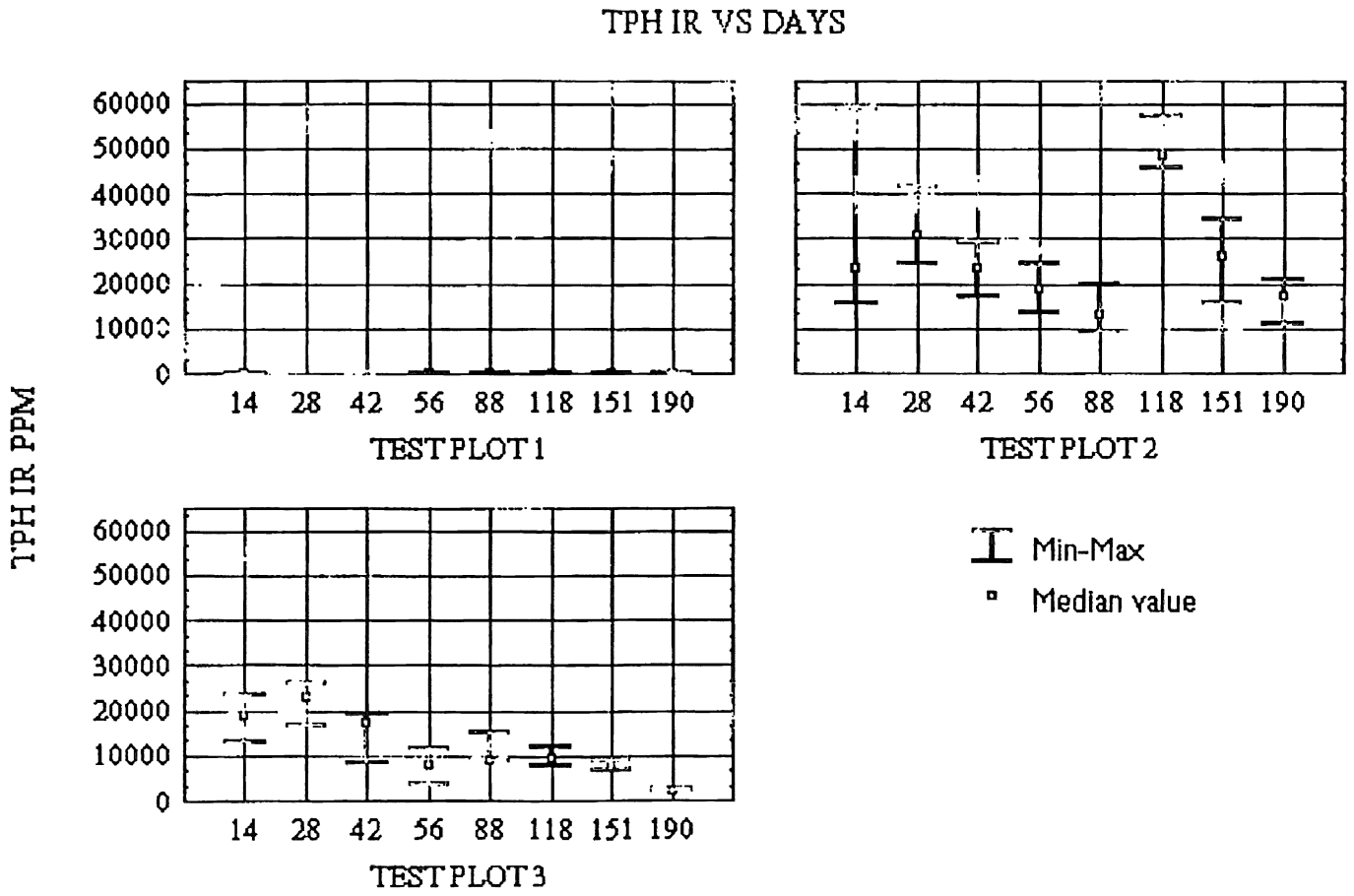


Figure 5. Total Petroleum Hydrocarbons by Infrared Spectrometry vs. Days for Test Plots Nos. 1-3

maximum value of 7,677 ppm TPH on day 190. In contrast to test plot No. 2, TPH concentrations in the deeper intervals of test plot No. 3 both reached their maximum values on day 14. The 1-2 ft interval had 5,562 ppm TPH and the 2-3 ft interval had 3,941 ppm TPH.

BTEX Content of Test Plot Soil

Table VI summarizes the distribution of benzene, toluene, ethylbenzene and xylenes in test plots No. 2 and No. 3 over the duration of the study. Figures 6-9 are BTEX concentrations from the 0-1 ft intervals for test plots No. 2 and No. 3. Test plot No. 1 is not

TABLE VI
BTEX CONCENTRATIONS TEST PLOTS 2 AND 3

		TEST PLOT 2				TEST PLOT 3			
DAY	DEPTH, FEET	BENZENE, PPM	TOLUENE, PPM	ETHYL BENZENE, PPM	XYLENE, PPM	BENZENE, PPM	TOLUENE, PPM	ETHYL BENZENE, PPM	XYLENE, PPM
14	1*	<0.50	16.42	32.02	122.5	<0.50	5.23	<0.50	69.68
	2	<0.50	1.99	2.28	12.14	<0.50	1.27	<0.50	14.47
	3	<0.50	<0.50	0.76	6.87	<0.50	1.65	<0.50	10.13
28	1*	<0.50	1.13	<0.50	28.55	<0.50	<0.50	<0.50	6.17
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
42	1*	<0.50	<0.50	3.12	49.25	<0.50	0.51	<0.50	6.47
	2	<0.50	<0.50	<0.50	1.54	<0.50	<0.50	<0.50	<0.50
	3	-	-	-	-	<0.50	<0.50	<0.50	0.64
56	1*	<0.50	<0.50	<0.50	50.15	<0.50	0.86	<0.50	1.27
	2	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
	3	<0.50	<0.50	<0.50	3.80	<0.50	<0.50	<0.50	<0.50
88	1*	<0.50	<0.50	<0.50	6.29	<0.50	<0.50	<0.50	<0.50
	2	<0.50	1.36	<0.50	4.35	<0.50	<0.50	<0.50	<0.50
	3	1.80	2.96	<0.50	6.12	<0.50	<0.50	<0.50	<0.50
118	1*	<0.50	0.57	<0.50	31.51	<0.50	<0.50	<0.50	<0.50
	2	<0.50	<0.50	<0.50	3.17	<0.50	<0.50	<0.50	<0.50
	3	<0.50	<0.50	<0.50	2.41	<0.50	<0.50	<0.50	<0.50
151	1*	<0.50	<0.50	<0.50	17.44	<0.50	<0.50	<0.50	<0.50
	2	<0.50	<0.50	<0.50	4.25	<0.50	0.52	<0.50	<0.50
	3	<0.50	<0.50	<0.50	2.20	<0.50	<0.50	<0.50	<0.50
190	1*	<0.50	<0.50	<0.50	11.64	<0.50	<0.50	<0.50	<0.50
	2	<0.50	<0.50	<0.50	3.35	<0.50	<0.50	<0.50	<0.50
	3	<0.50	<0.50	<0.50	2.20	<0.50	<0.50	<0.50	<0.50

Note: * median value of four sample measurements.

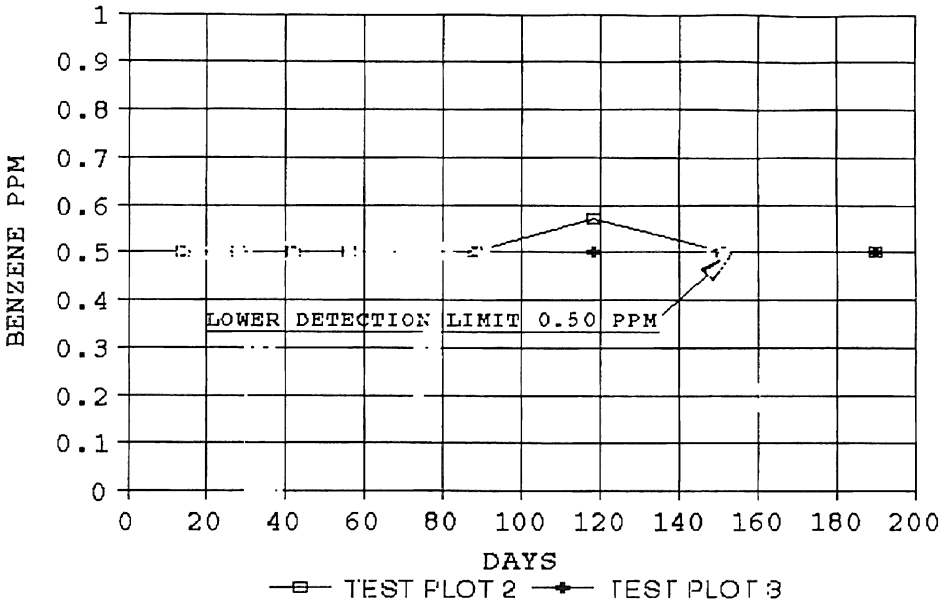


Figure 6. Benzene ppm vs. Days, Test Plots 2 and 3

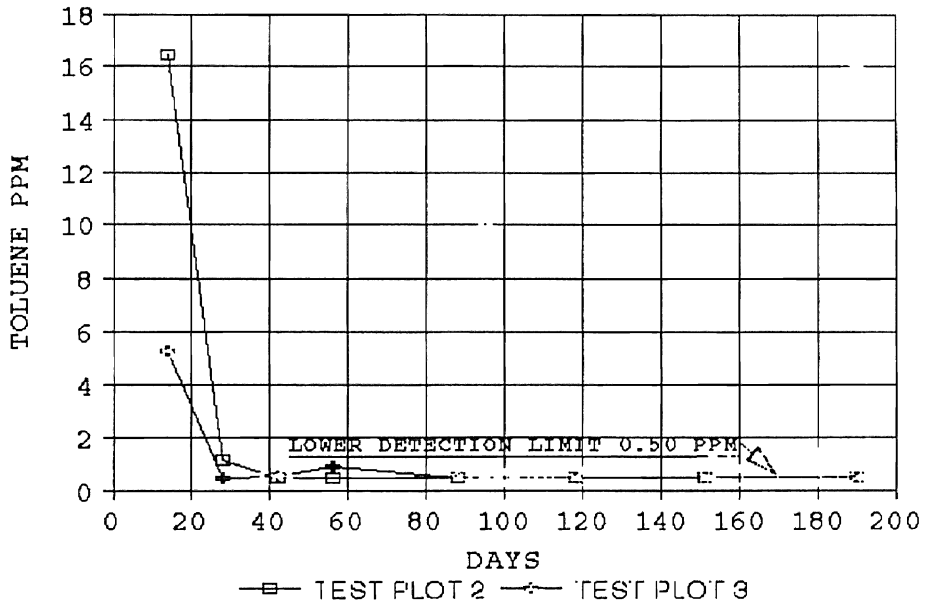


Figure 7. Toluene ppm vs. Days, Test Plots 2 and 3

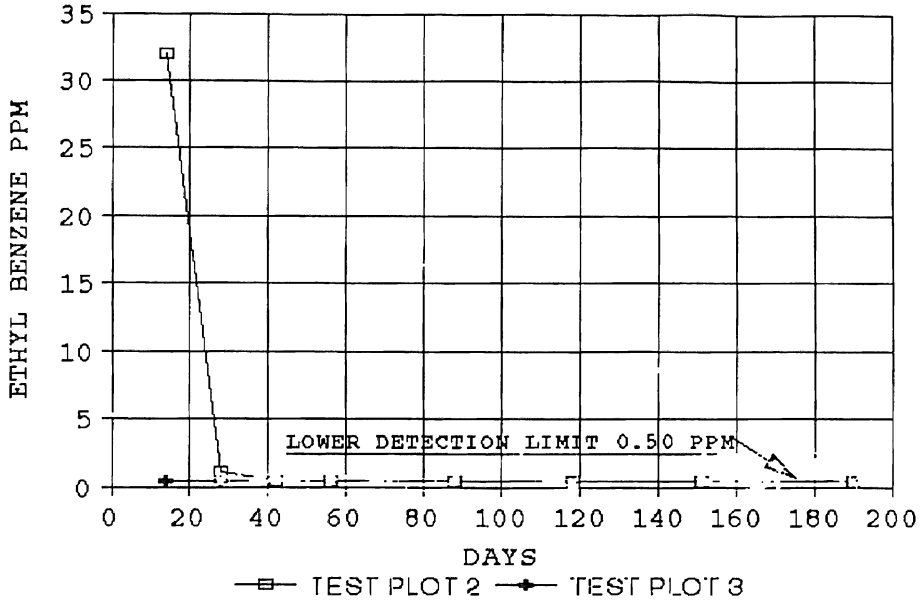


Figure 8. Ethylbenzene ppm vs. Days, Test Plots 2 and 3

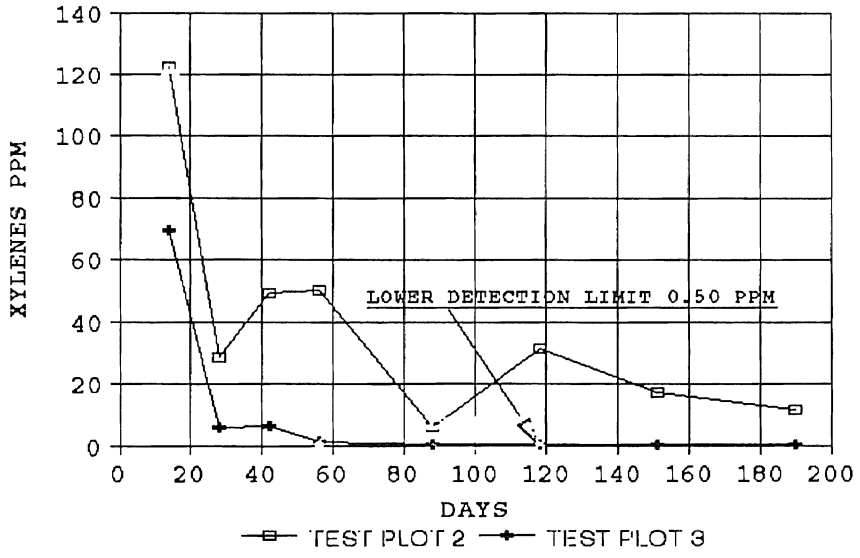


Figure 9. Xylenes ppm vs. Days, Test Plots 2 and 3

considered in this presentation because all measured BTEX values are at the 0.50 ppm detection limit and indicates that by day 14, all benzene concentration had been reduced to at or near the 0.50 ppm detection limit in both test plots No. 2 and No. 3. Figure 7 shows that toluene is reduced to the 0.50 ppm detection limit in test plot No. 2 by day 42 and in test plot No. 3 by day 28. Ethylbenzene is reduced to detectable limit in test plot No. 2 by day 56 and in test plot No. 3 by day 14. Xylenes were reduced to the detection limit only in test plot No. 3. Xylenes concentration was not reduced to detection limit in test plot No. 2 during the duration of the experiment. This fact makes xylenes the only BTEX component that shows a difference between treatment options. Raterman *et al.* (1993) concluded from a microcosm study of Michigan Silurian crude oil contaminated soil that BTEX removal was primarily the result of volatilization. Vapor pressures of benzene (45.43 mm Hg), toluene (12.43 mm Hg) and ethyl benzene (3.77 mm Hg) support this conclusion and explain the relative loss rates of these three BTEX components observed in this experiment. Xylenes' vapor pressure (3.26 mm Hg) does not support volatilization as an explanation of the loss differences observed in test plot No. 2 between ethyl benzene and xylenes. Biomass development studies conducted by Corseul and Weber (1993) show that the time needed to develop microorganisms required to remediate xylenes is longer than the time needed to develop microorganisms that will remediate other BTEX components. It is concluded that the observed differences between xylenes and ethyl benzene concentrations in test plots No. 2 and No. 3 are a function of bioremediation.

Microtox® Acute Toxicity of Soil WSF

WSF extracts from 122 soil samples were tested for toxicity using the Microtox® Basic Protocol. The basic protocol as conducted in this study is outlined in Appendix E. The results by sample of these tests are presented in Appendix D, Table XI. Table VII summarizes the five minute EC50, EC20, EC10, and EC1 values for test plots 1, 2 and 3.

TABLE VII

MICROTOX® FIVE MINUTE EFFECTIVE CONCENTRATIONS % WSF

TEST PLOT 1, 2 AND 3

DAY	DEPTH FEET	TEST PLOT 1				TEST PLOT 2				TEST PLOT 3			
		EC50	EC20	EC10	EC1	EC50	EC20	EC10	EC1	EC50	EC20	EC10	EC1
14	1*	187.7	29.0	10.5	1.10	22.9	8.9	6.0	1.6	31.4	7.4	3.2	0.29
	2					225.0	17.3	3.8	0.04	45.7	17.4	9.9	1.90
	3					83.0	5.2	1.0	0.01				
28	1*					27.5	0.74	0.15	.001	391.9	54.6	18.6	1.30
	2												
	3												
42	1*					40.0	5.7	1.9	0.03	49.1	22.8	13.9	4.03
	2									63.1	12.0	4.5	0.25
	3									226.7	34.7	11.6	0.45
56	1*	214.4	31.7	9.1	0.3	42.3	7.1	2.3	0.07	101.6	41.1	16.7	1.20
	2	510.4	70.7	22.3	0.7	485.4	45.7	11.5	0.19	353.3	95.2	44.2	4.6
	3	358.2	42.3	12.1	0.3	316.4	35.4	9.8	0.22	434.4	41.3	10.4	0.18
88	1*	10,771	456.6	88.0	1.5	28.9	4.4	1.1	0.02	26.1	13.1	8.3	1.9
	2	188.2	44.0	18.8	1.5	33.7	15.2	9.5	2.4	45.8	22.2	14.5	4.2
	3	269.3	72.4	33.6	3.5	14.4	1.6	0.45	0.01	118.4	41.5	22.5	3.7
118	1*	785.1	605.9	48.1	1.9	6.4	0.81	0.24	0.01	143.7	45.3	23.1	2.7
	2	10,526	751.2	160.5	1.7	61.8	7.4	2.2	0.06	239.7	49.6	19.7	1.3
	3	1,110	182	63.2	2.8	33.6	3.7	1	0.02	498	40.3	9.2	0.10
151	1*	4,199	379.4	93.1	1.5	26.2	6.0	2.5	0.2	52.8	21.1	16.3	3.7
	2	8,996	543.2	105.2	0.9	355.1	32.2	7.9	0.12	159.3	56.2	30.5	5
	3	1,709	208.3	60.8	1.6	243.5	18.8	4.2	0.05	298.9	87.4	52.6	5.1
190	1*	11,395	743.5	168.6	3.3	21.8	2.8	0.84	0.02	170	44.1	20.7	2.10
	2	51,563	1,784	249	0.7	81.2	8.4	2.2	0.04	350.7	85.7	37.6	3.3
	3	11,934	800.8	164.9	1.5	83	17.8	7.2	0.51	852.7	126.2	41.3	1.51

Note: * median value of four samples

EC50% WSF concentrations of the uncontaminated test plot No. 1 upper one-foot interval ranged from a minimum of 187% to a maximum of 11,395%. Values of effective concentration over 100% WSF are presented to provide a measure of relative toxicity and are the result of extrapolations to concentrations well above the maximum 45% concentration used in the Microtox® basic protocol. Concentrations furnished by these

extrapolations are the mid-point of a very broad 95% confidence intervals, and their accuracy is therefore suspect. Inspection of confidence intervals determined that the EC10 metric provided the best value for description of toxicity of uncontaminated soil during the 190-day duration of the experiment. EC50% WSF concentrations of the contaminated test plot No. 2 upper one foot interval proved to be a good descriptor of toxicity for the entire study. Effective concentrations of WSF from amended test plot No. 3 exhibited high toxicities during early sampling events were best described by EC50. Low toxicities measured during later sampling events were found to be best interpreted by EC20 or EC10. The less toxic two and three feet intervals of both test plots No. 2 and No. 3 exhibited this change at some point in the experiment.

Figures 10-13 are graphs of effective concentrations (EC50, EC20, EC10 and EC1) vs. days which summarize measured toxicity ranges from the upper one-foot interval of test plots No. 2 and No. 3. Inspection of Figure 10 graph of EC50 vs. days indicates that on day 14, the range of toxicity of test plot No. 3 falls within the range of toxicity measured for test plot No. 2. By day 28, test plot No. 2 median toxicity as gauged by EC50 has reduced only 4.6% WSF, while during the same period, median toxicity of test plot No. 3 had reduced by 360% WSF. EC50 values on day 42 for test plot No. 2 continued to display high variability from 9.46% WSF to 160.23% WSF with a median value of 40% WSF. From day 28 to day 42, test plot No. 3 displayed an increased toxicity to a median value of 49.1%WSF. Toxicity measurements from test plot No. 3 showed a much narrower range of variability (38.13% to 67.41%) than the toxicity of test plot No. 2. This decreased variability is a function of tilling test plot No. 3 which dispersed toxicants evenly within the plot.
Figures 10-13 all demonstrate fluctuations in effective concentrations which may be accounted for by measured concentrations of TPH.

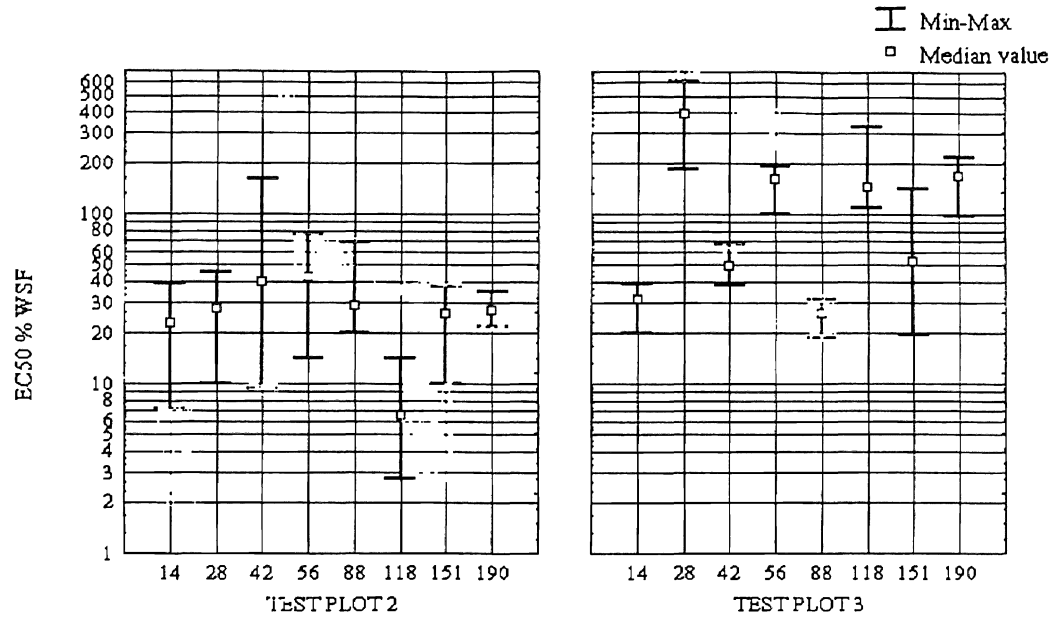


Figure 10. Microtox® Basic Protocol EC50% WSF vs. Days for One-Foot Interval, Test Plots 2 and 3

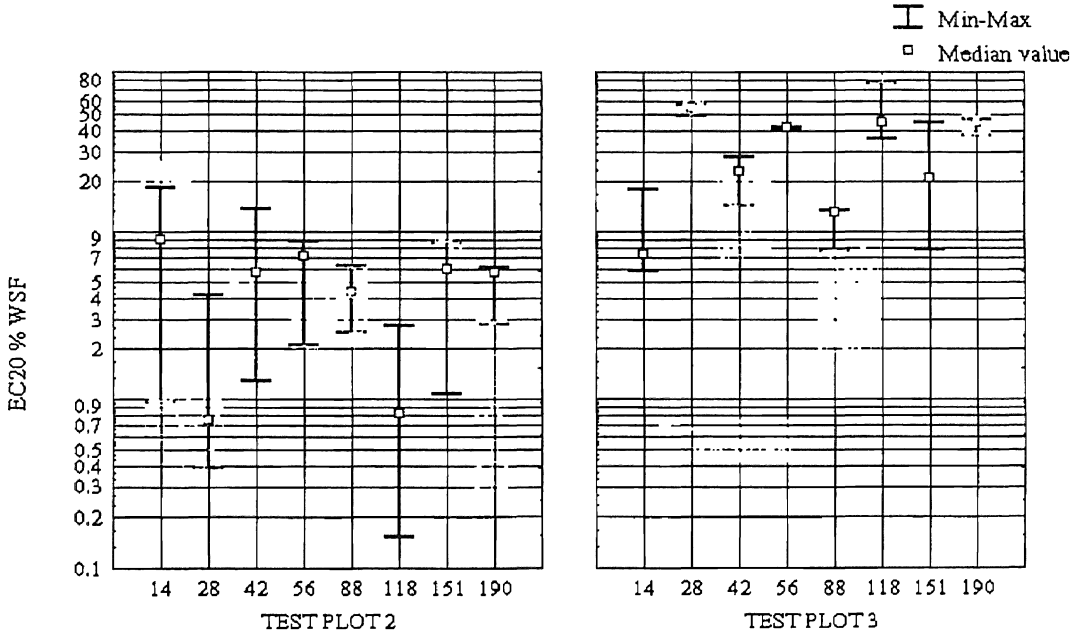


Figure 11. Microtox®Basic Protocol EC20% WSF vs. Days for One-Foot Interval, Test Plots 2 and 3

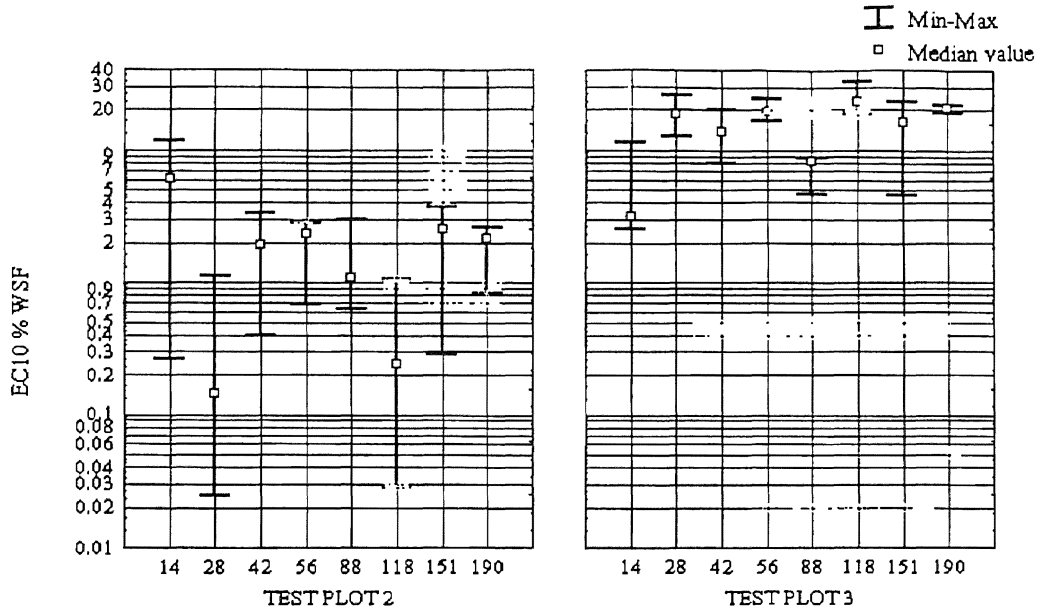


Figure 12. Microtox® Basic Protocol EC10% WSF vs. Days
for One-Foot Interval, Test Plots 2 and 3

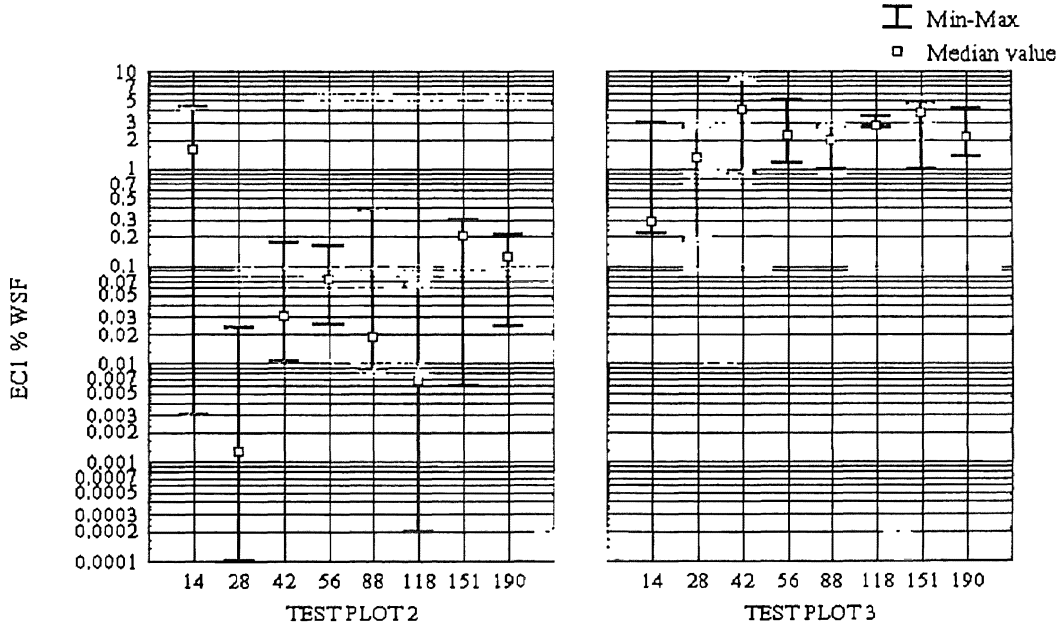


Figure 13. Microtox® Basic Protocol EC1% WSF vs. Days
for One-Foot Interval, Test Plots 2 and 3

Microtox® Effective Concentrations and TPH Concentration

To determine if variations in effective concentration could be correlated with TPH variations, graphs of EC values versus days were constructed for each plot and each effective concentration metric (EC50, EC20, EC10 and EC1). Figure 14 summarizes Microtox® effective concentrations and TPH IR ppm vs. days for the 0-1 foot interval of uncontaminated test plot No. 1. Median TPH IR concentrations measured in the uncontaminated test plot No. 1 ranged from 58 ppm to 14 ppm. Analysis of graphs in Figure 14 reveals that Microtox® effective concentration changes do not correlate to changes in TPH concentration over this small interval. Correlation would be marked by convergence and divergence of TPH and effective concentrations. Figure 15 summarizes Microtox® effective concentrations and TPH IR ppm vs. days for the 0-1 foot interval of crude oil contaminated test plot No. 2. Median TPH IR concentrations measured in the contaminated test plot No. 2 ranged from 48,435 ppm down to 13,215 ppm. The effective concentration as measured by all metrics was, at a minimum, at the high TPH concentration registered on day 118. At the minimum TPH concentration, none of the effective concentration metrics recorded a maximum value. Figure 16 summarizes Microtox® effective concentrations and TPH IR ppm vs. days for the 0-1 foot interval of crude oil contaminated test plot No. 3. Median TPH concentrations for test plot No. 3 varied from a maximum of 22,634 ppm on day 28 to a minimum of 2,209 ppm on day 190. Microtox® effective concentrations measured in test plot No. 3 show the least correlation between toxicity and TPH concentration. This is best illustrated by the fact that the WSF from the soil sample with the maximum TPH recorded the highest effective concentrations. Figures 17-19 are graphs of effective concentrations versus TPH IR for each test plot. The low correlations between TPH concentrations and effective concentrations emphasizes the fact that a singular effective concentration does not provide a means for tracking remediation.

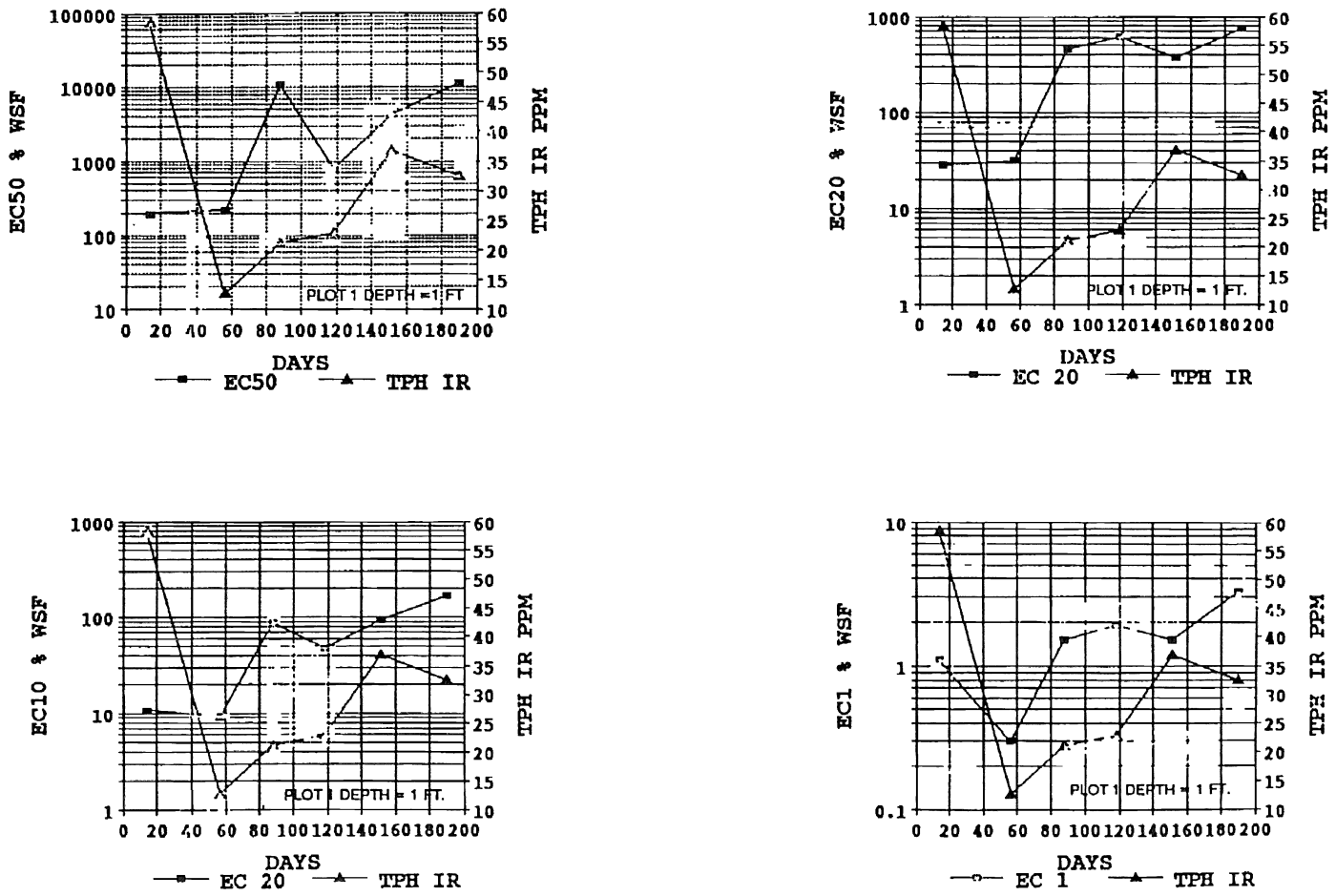


Figure 14. Test Plot No. 1. Effective Concentrations and TPH IR vs. Days

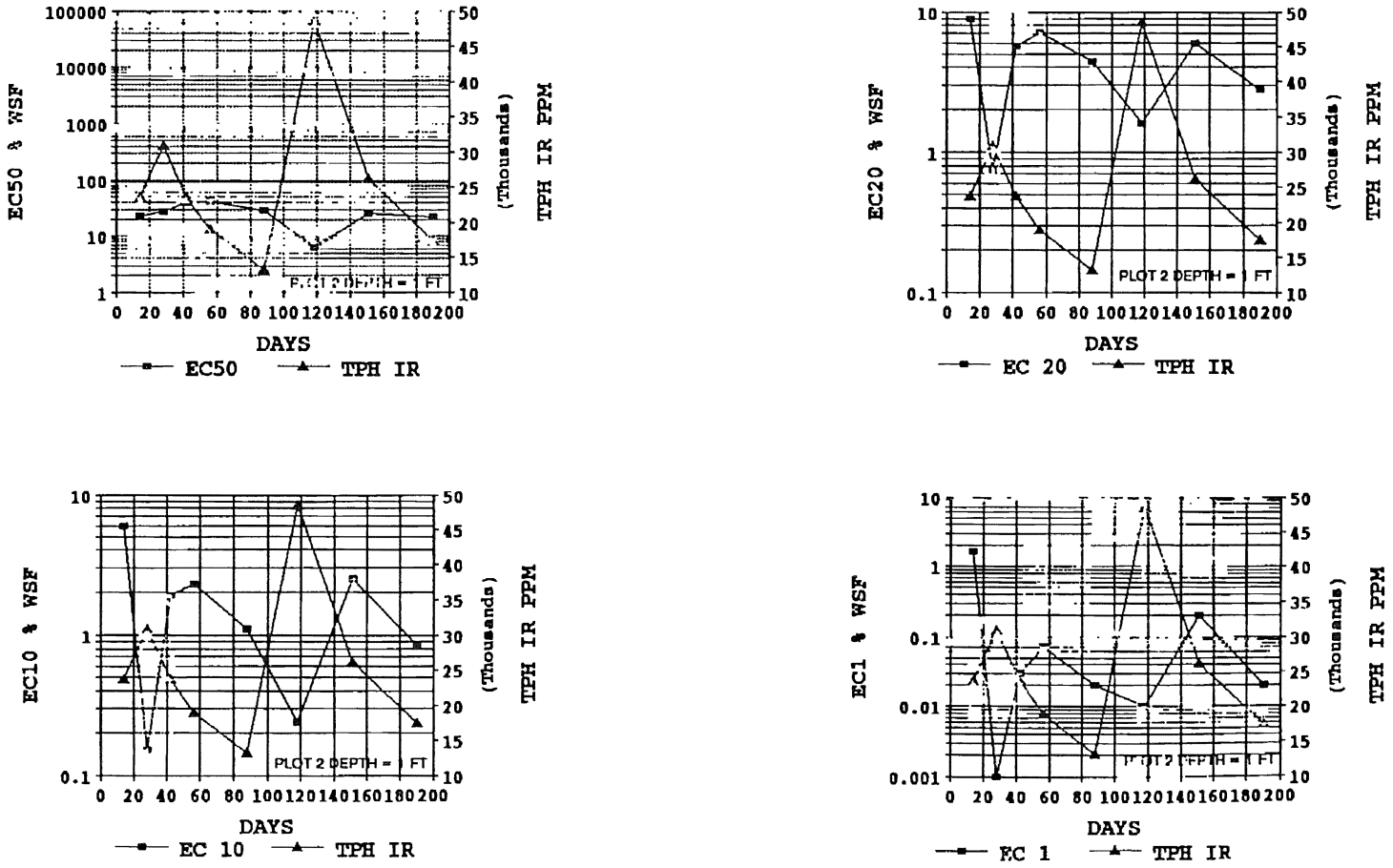


Figure 15. Test Plot 2. Effective Concentrations and TPH IR vs. Days

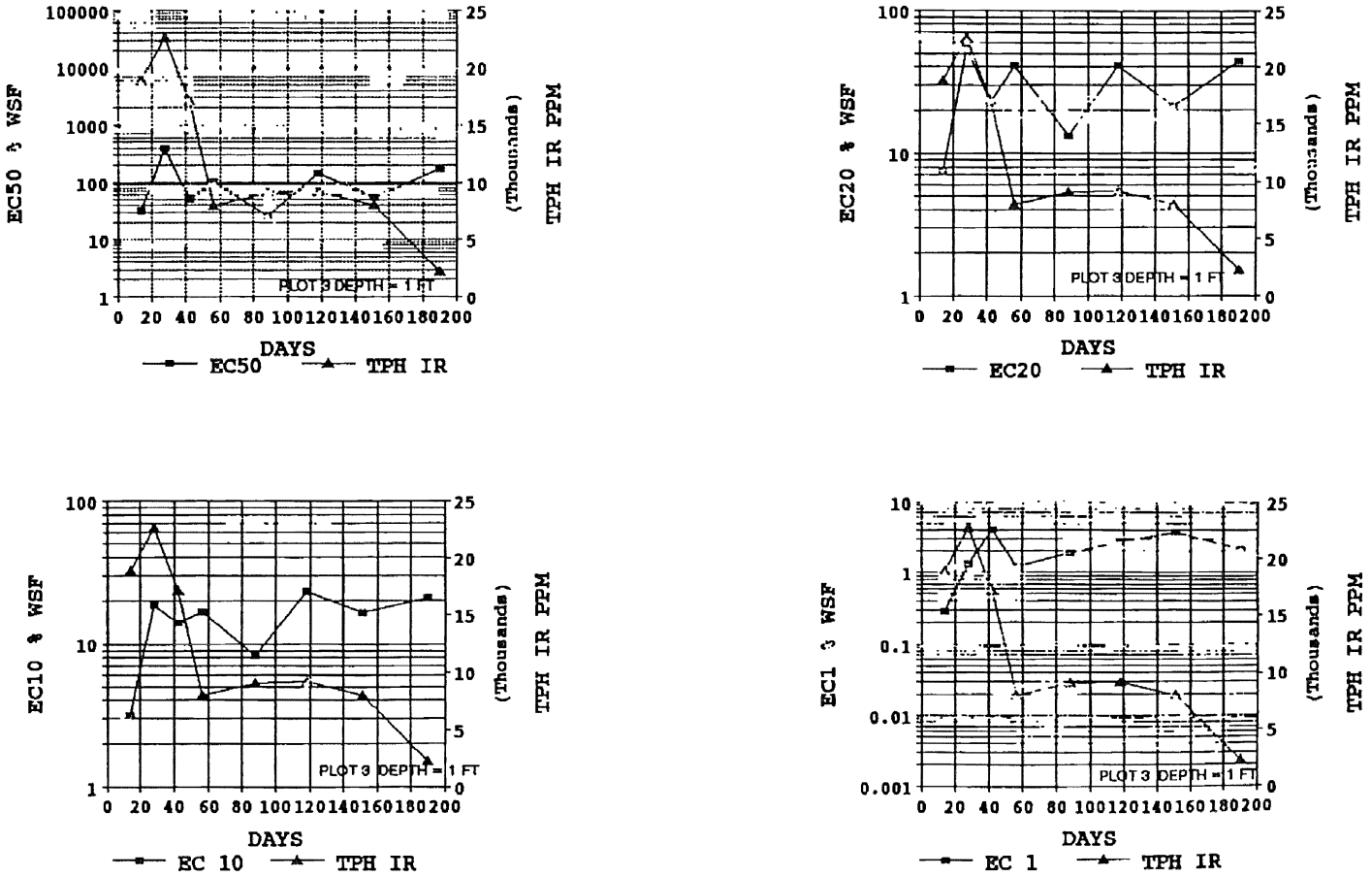
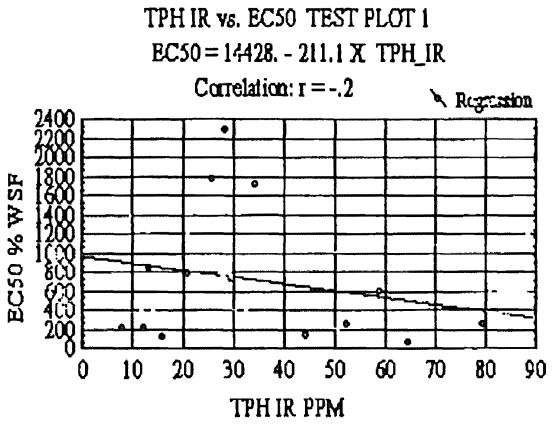
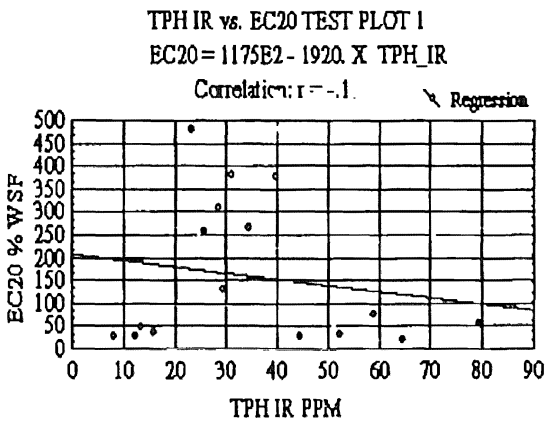
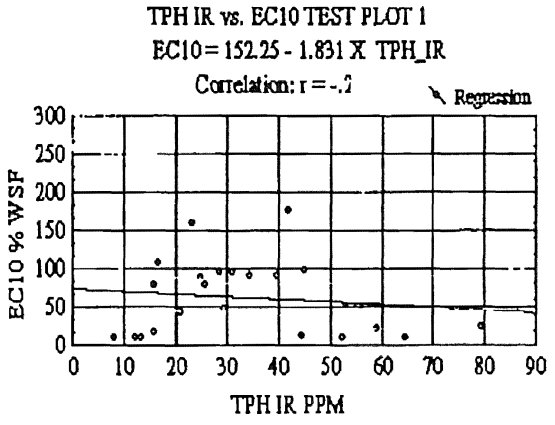


Figure 16. Test Plot 3. Effective Concentrations and TPH IR vs. Days







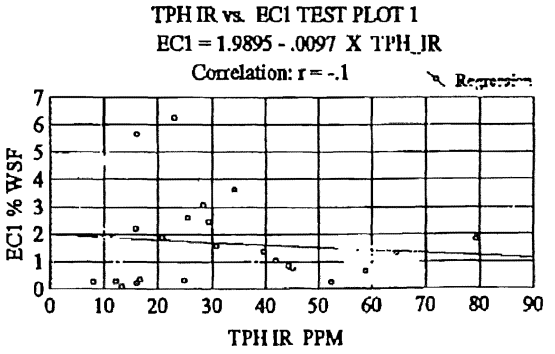


Figure 17. Effective Concentrations vs. TPH IR ppm, Test Plot 1

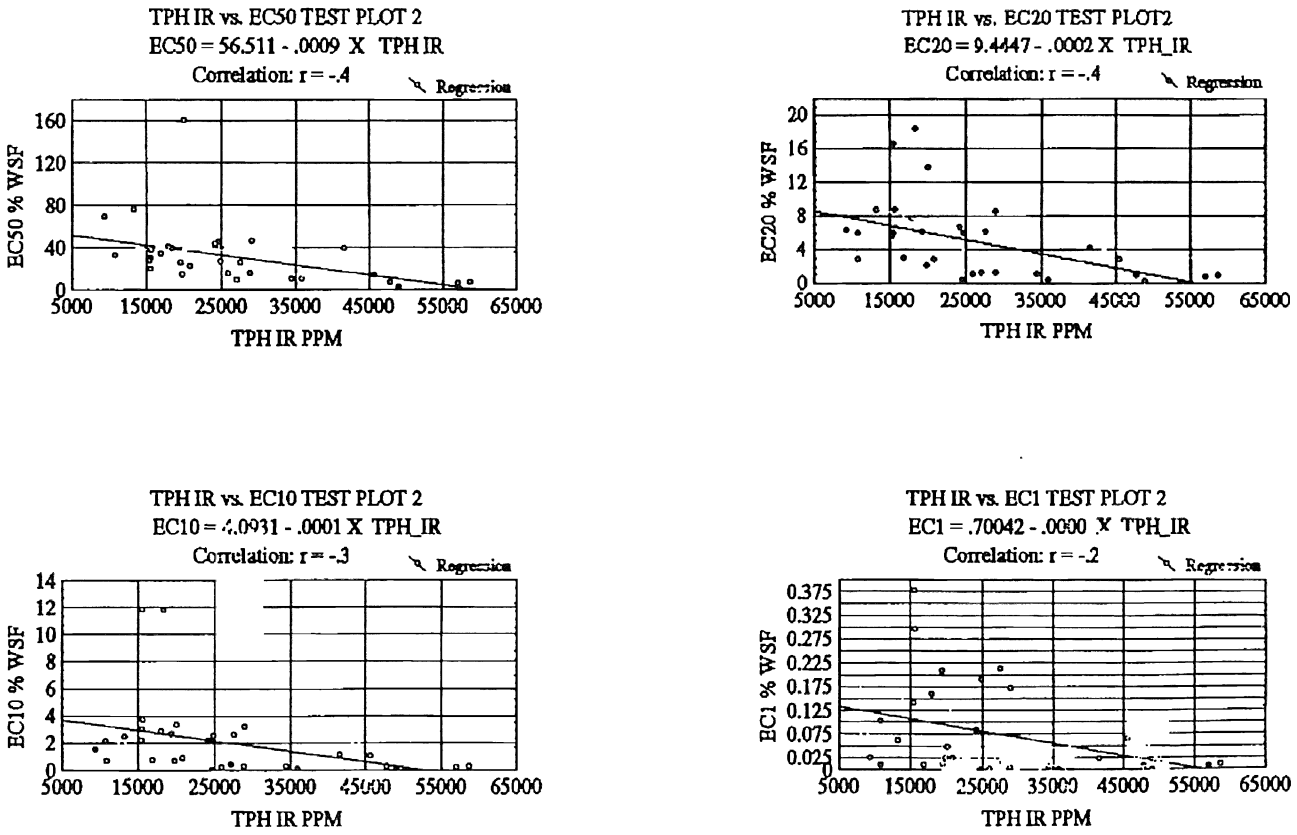


Figure 18. Effective Concentrations vs. TPH IR ppm, Test Plot 2

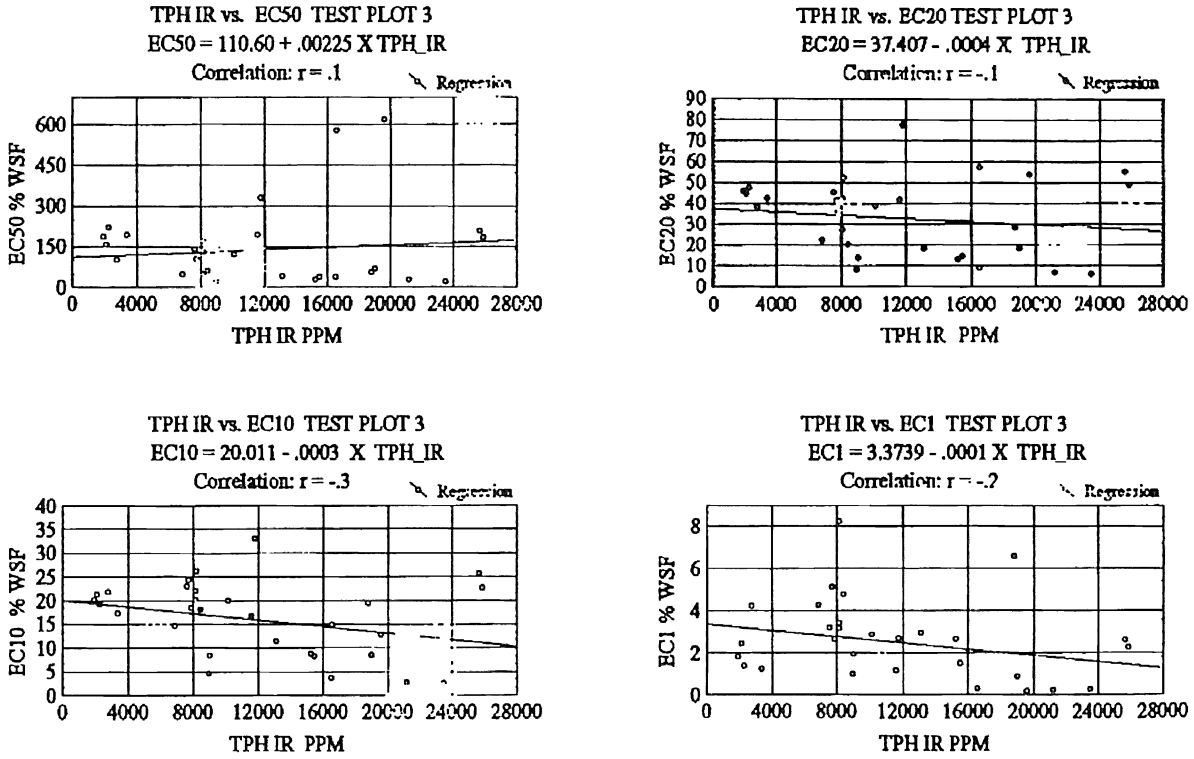


Figure 19. Effective Concentrations vs. TPH IR ppm, Test Plot 3

Microtox® Reagent Light Inhibition

During the soil remediation being monitored in this study, the *effective* concentration values of the WSF measured by the Microtox® basic protocol ranged extensively. At different times or in different test plots, it was found that different effective concentration metrics (EC50, EC20, EC10 or EC1) provided the value with the smallest confidence interval or was the best estimator of toxicity. The smallest confidence interval falls at the mean of the basic protocol data and does not necessarily coincide with any of the above effective concentrations. The Microtox® basic protocol as used in this study measured the light inhibition caused by 45%, 22.5%, 11.25% and 5.65% dilutions of the WSF compared to a blank. The mean of these data occurs at 19.7% (approximate 20%). The graph of Log Gamma (Γ) vs. Log Concentration can be used to determine light inhibition for any given concentration of WSF. The 20% concentration provides a value which is easily plotted on a Log Gamma (Γ) v. Log Concentration graph at the approximate mean of the data. The light inhibition percentage caused by the 20% dilution of WSF is calculated by determining the gamma (Γ) value for the 20% dilution. This gamma value is reduced to a light inhibition % value by the following equation (2):

$$\% \text{ Inhibition} = \left(1 - \frac{1}{\Gamma + 1}\right) \times 100 \qquad (2)$$

Table VIII summarizes Microtox® light inhibition % caused by a 20% WSF. Table XI in Appendix F reports Microtox® light inhibition for each soil sample extract measured in this study. Figures 20- 22 are graphs of inhibition of 20% WSF and TPH concentrations versus days for test plots Nos. 1-3 respectively. Each graph summarizes the data for the upper one-foot interval of each test plot as presented in Table VIII.

Microtox® No Observed Effects Concentration and Solid Phase Toxicity Tests

Microtox® No Observed Effects Concentration (NOEC) of WSF and Large Sample Solid Phase Toxicity (LSPT) and Basic tests were performed on soil samples collected on

TABLE VIII

MICROTOX® REAGENT LIGHT INHIBITION % CAUSED BY A 20% WSF

		TEST PLOT 1		TEST PLOT 2		TEST PLOT 3	
DAY	DEPTH FEET	INHIB %	TPHIR PPM	INHIB %	TPHIR PPM	INHIB %	TPHIR PPM
	1*	16	59	40	23,635	38	18,871
14	2			21	5,975	26	5,561
	3			33	3,925		3,941
	1*			49	30,912	11	[illegible]
28	2						
	3						
	1*			37	23,628	16	17,141
42	2			32	4,389	22	5,147
	3			15	3,584	15	3,089
	1*	15	13	32	18,923	10	7,937
56	2	17	19	13	2,061	5	546
	3	134	21	15	36	15	7,962
	1*	5	21	45	13,215	6	9,035
88	2	11	12	29	9,470	17	3,560
	3	6	10	55	4,676	8	836
	1*	6	23	68	48,435	9	9,152
118	2	4	19	32	2,773	11	1,108
	3	4	18	41	3,394	15	1,039
	1*	4	37	44	26,232	10	8,017
151	2	5	56	16	3,910	5	2,741
	3	5	39	21	2,520	5	756
	1*	3	32	36	17,398	10	2,209
190	2	3	25	17	7,394	8	183
	3	3	17	22	7,677	8	104

day 190 of the study. The results of these tests are presented in detail in Table XII of Appendix G. Detailed NOEC and LSPT protocols as performed in this study are presented in Appendix E. The LSPT and NOEC protocols proved to be very labor, time and supply intensive protocols relative to the Basic protocol. For this reason, these protocols were performed only on samples taken during the day 190 sampling event to determine how they can best be used in a remediation monitoring project. Figures 23-25 display results of Microtox® Basic, LSPT and NOEC protocols performed on day 190 samples. A cursory

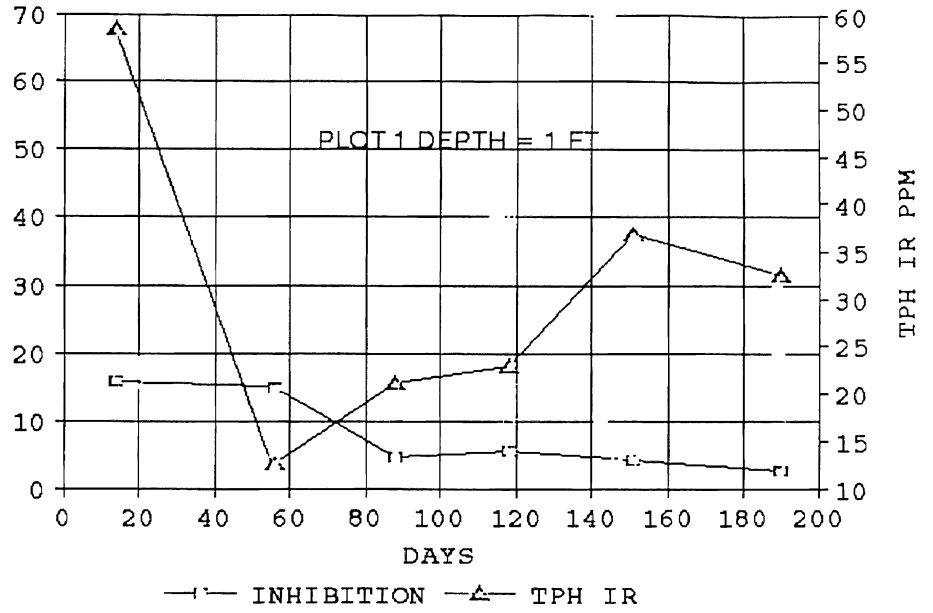


Figure 20. Microtox® Reagent Light Inhibition and TPH IR vs. Days, Test Plot 1

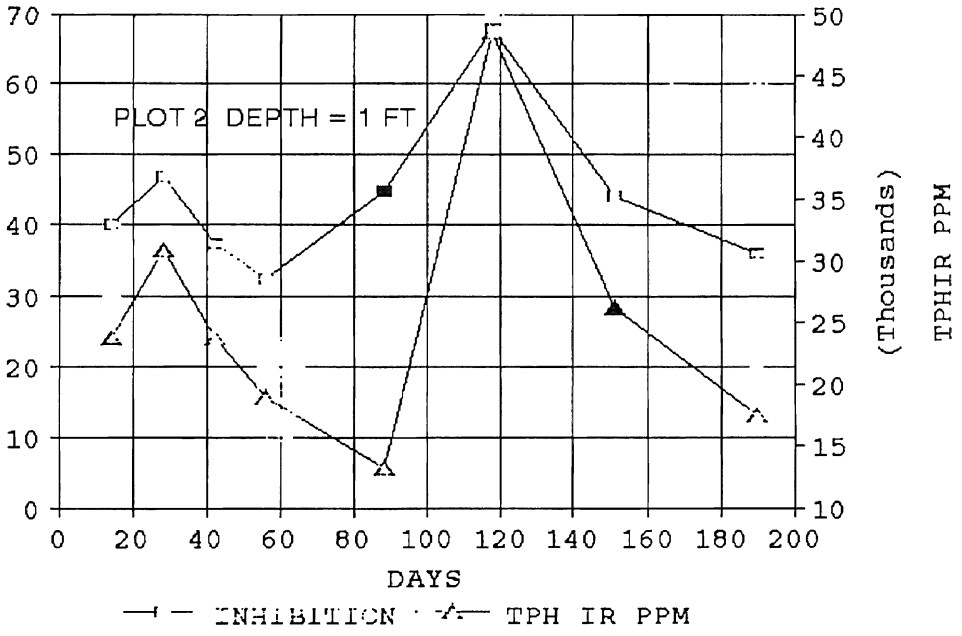


Figure 21. Microtox® Reagent Light Inhibition and TPH IR vs. Days, Test Plot 2

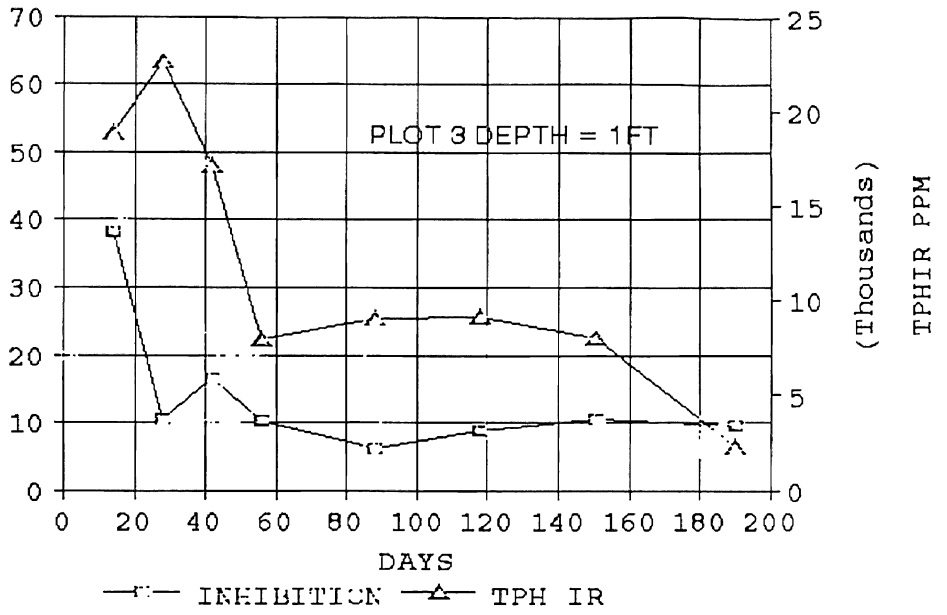


Figure 22. Microtox® Reagent Light Inhibition and TPH IR vs. Days, Test Plot 3

inspection of these results reveals that all three protocols provide measures of relative toxicity which are in agreement. Microtox® Basic, LSPT and NOEC protocols all show that uncontaminated test plot No. 1 has low toxicity. NOEC measurements of test plot No. 1 WSF extracts of 99.04% indicate that dilutions used in Basic protocol do not cause a Microtox® reagent light inhibition statistically different than the control. The LSPT protocol measurements of uncontaminated test plot No. 1 soil establish a median sediment contact toxicity EC50 of 2.08% compared to a 0.149% in test plot No. 2 and 0.545% in test plot No. 3. The NOEC protocol measurements of uncontaminated test plot No. 1 soil WSF had a median concentration of 99.04% compared to a 0.25% in test plot No. 2 and 32.19% in test plot No. 3. The basic protocol measurements of uncontaminated test plot No. 1 soil WSF measured a median EC50 of 11,395% compared to a 26.95% in test plot No. 2 and 170.04% in test plot No. 3. All three protocols point to the fact that test plot No. 2 is most toxic followed by test plot No. 3 and test plot No. 1 in order of decreasing toxicity.

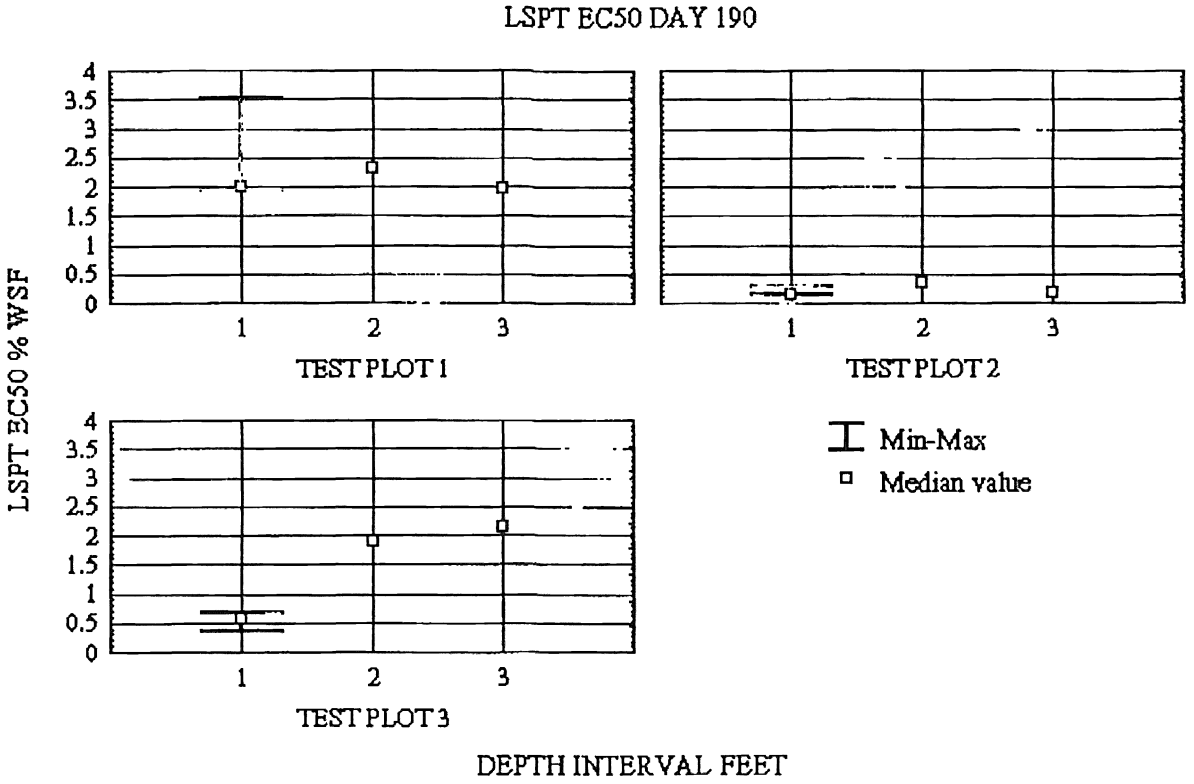


Figure 23. LSPT EC50 of Test Plots 1-3, Day 190

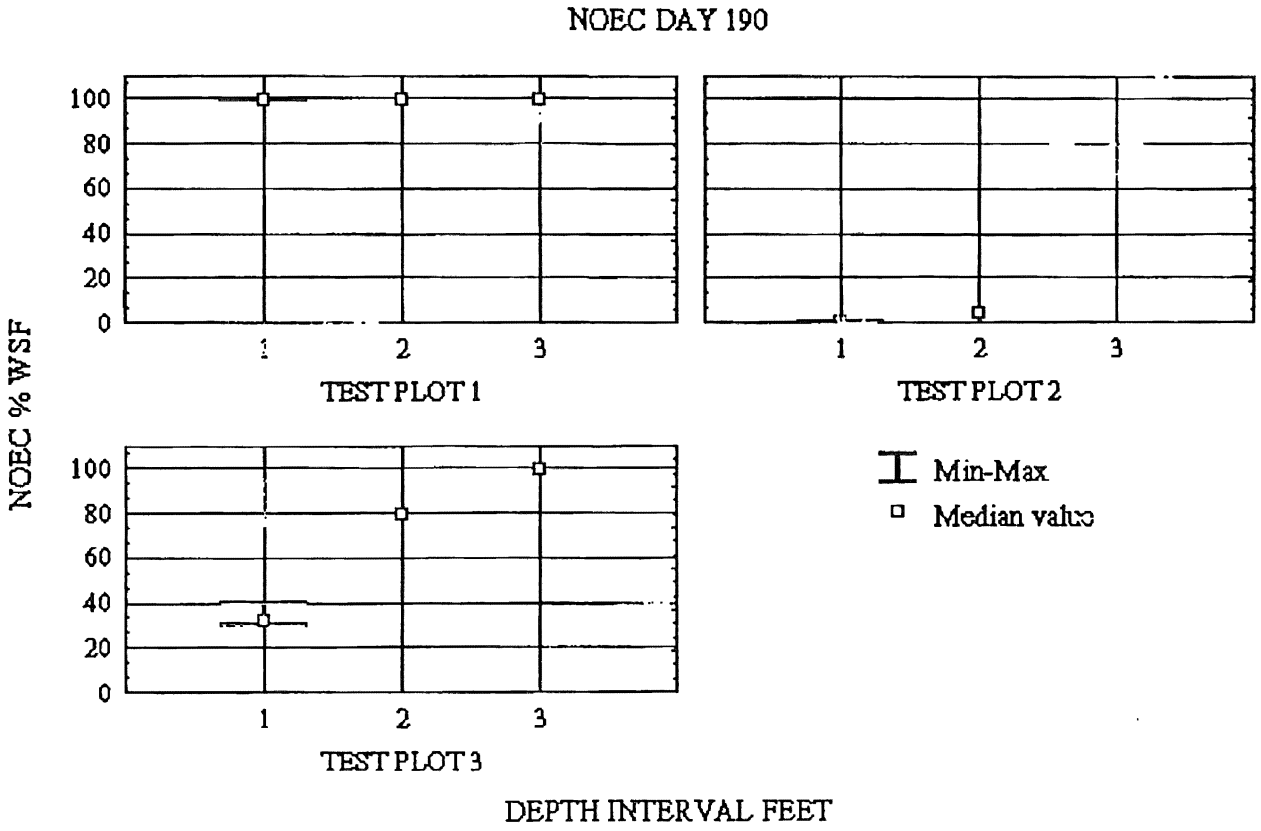


Figure 24. LSPT EC50 of Test Plots 1-3, Day 190

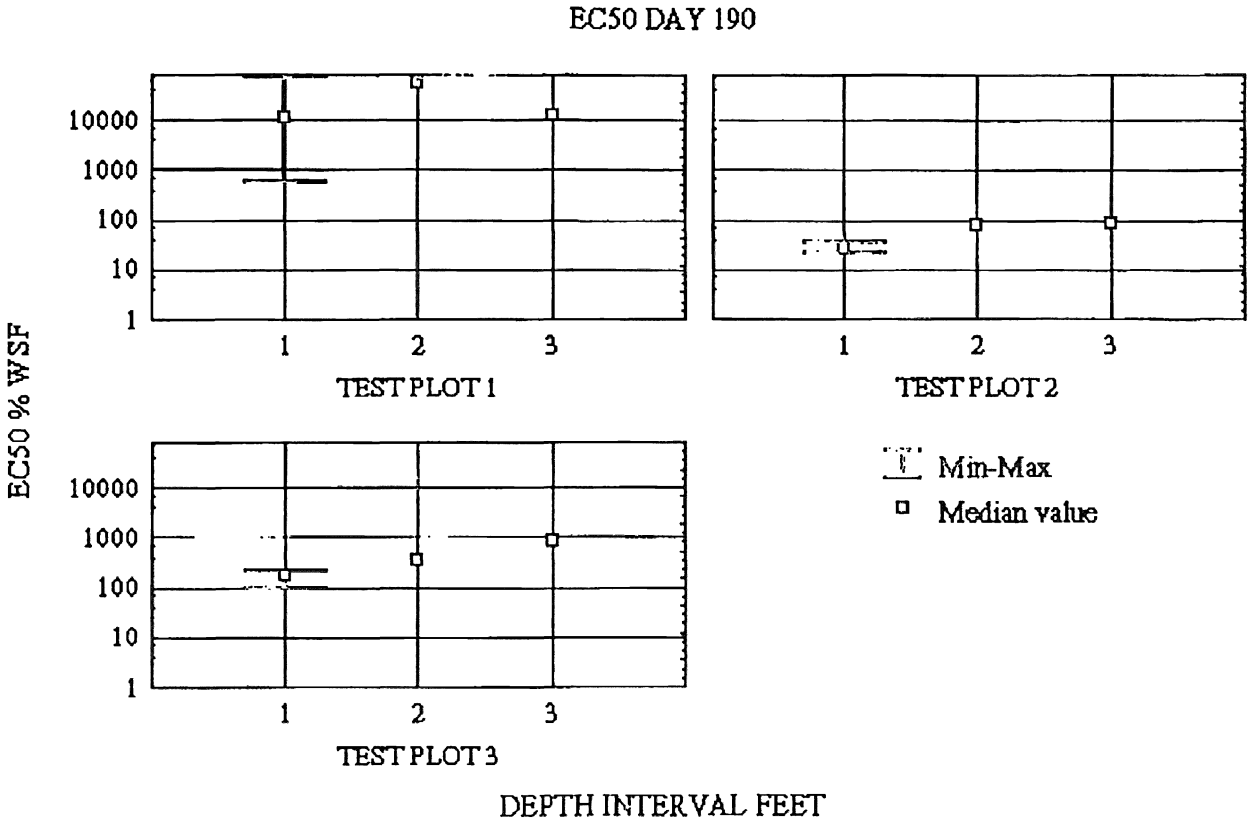


Figure 25. EC50 % WSF from Basic Protocol, Test Plots 1-3, Day 190

Figures 26-28 are graphs of TPH IR vs. LSPT %, NOEC % WSF and EC50 % WSF, respectively. All three graphs serve to reiterate the fact that the uncontaminated soil of test plot No. 1 is the least toxic followed by test plot No. 3 and test plot No. 2 in order of increasing toxicity. The graphs all show that high toxicity measurements are indicative of high soil TPH IR concentrations, but that toxicity is not related directly to TPH IR concentration.

Table IX summarizes the results discussed above.

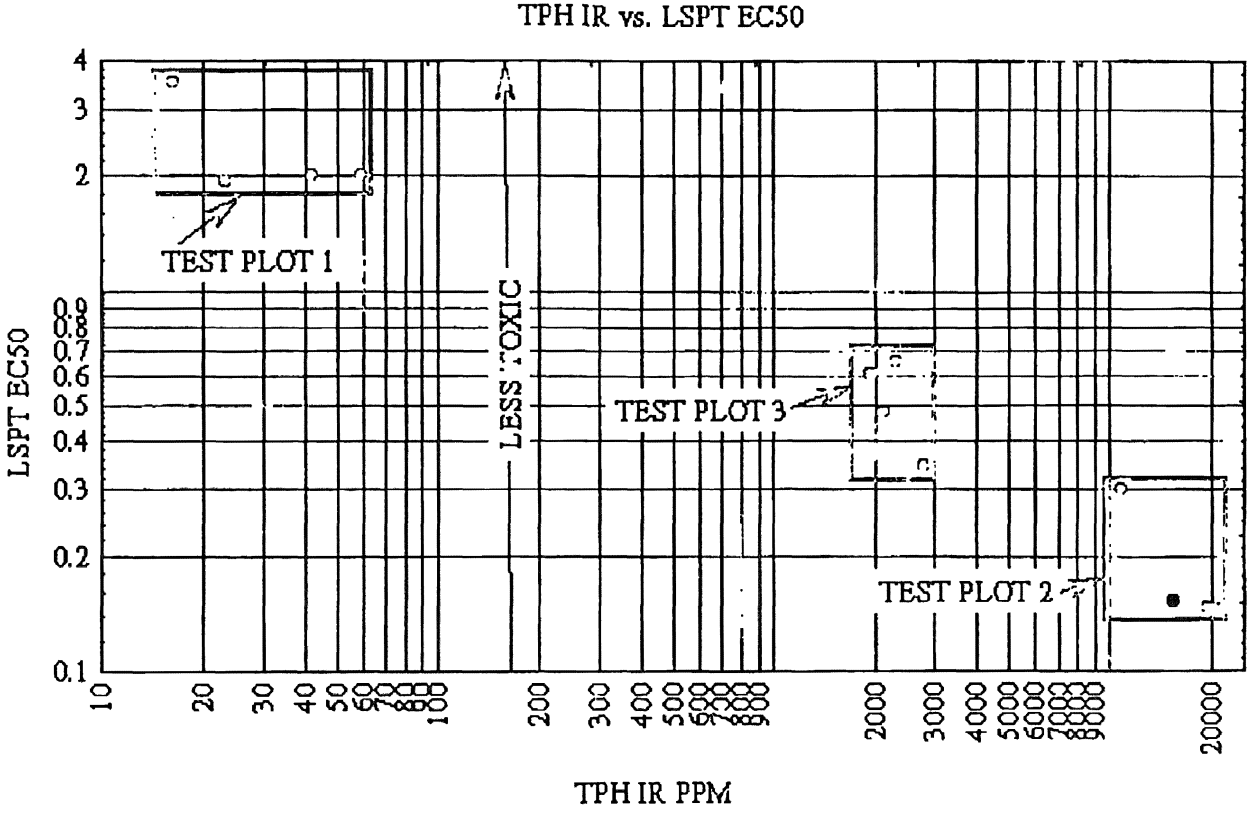


Figure 26. TPH IR vs. LSPT EC50, Test Plots 1-3, Day 190

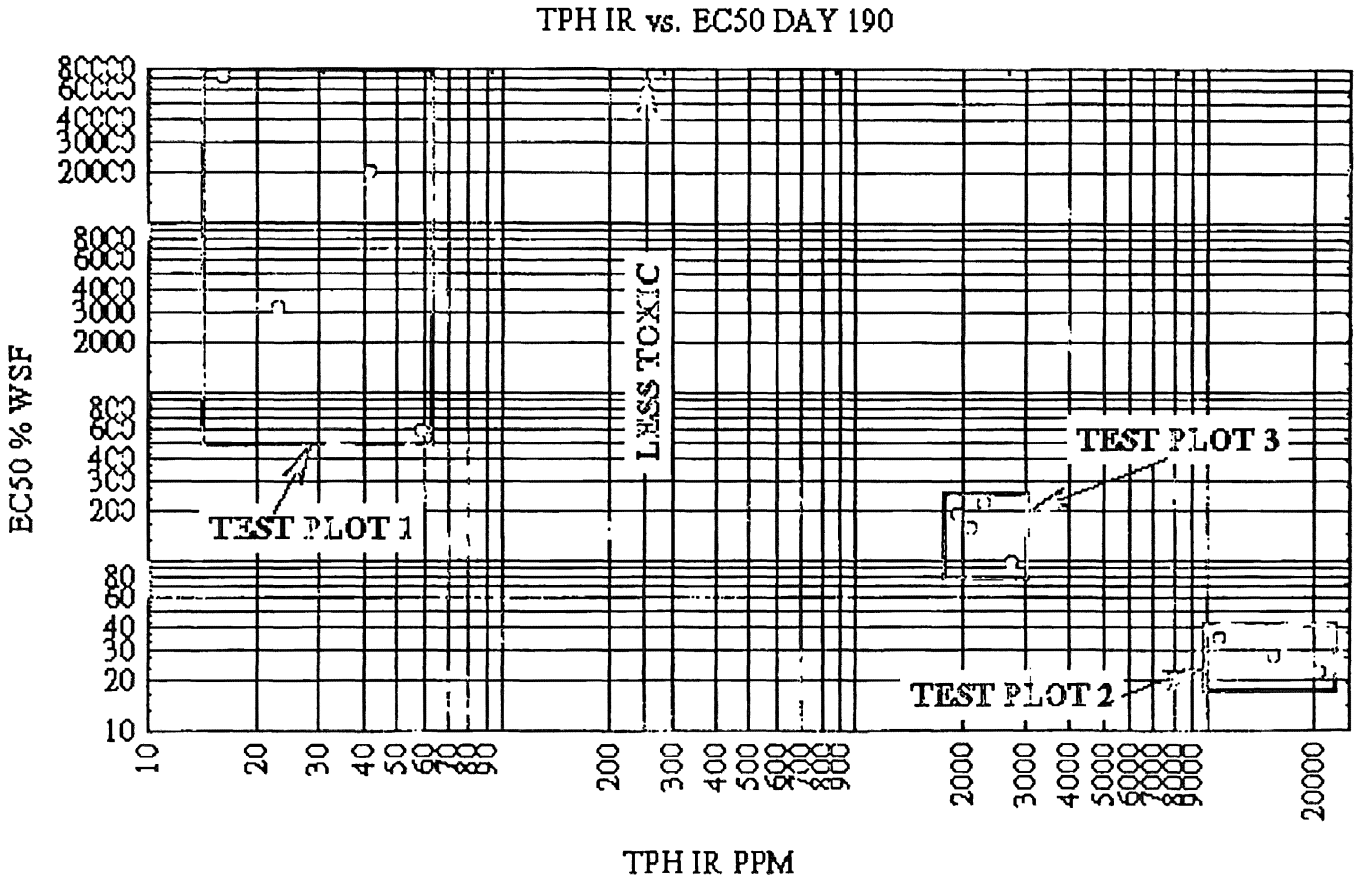


Figure 27. TPH IR vs. EC50 % WSF, Test Plots 1-3, Day 190

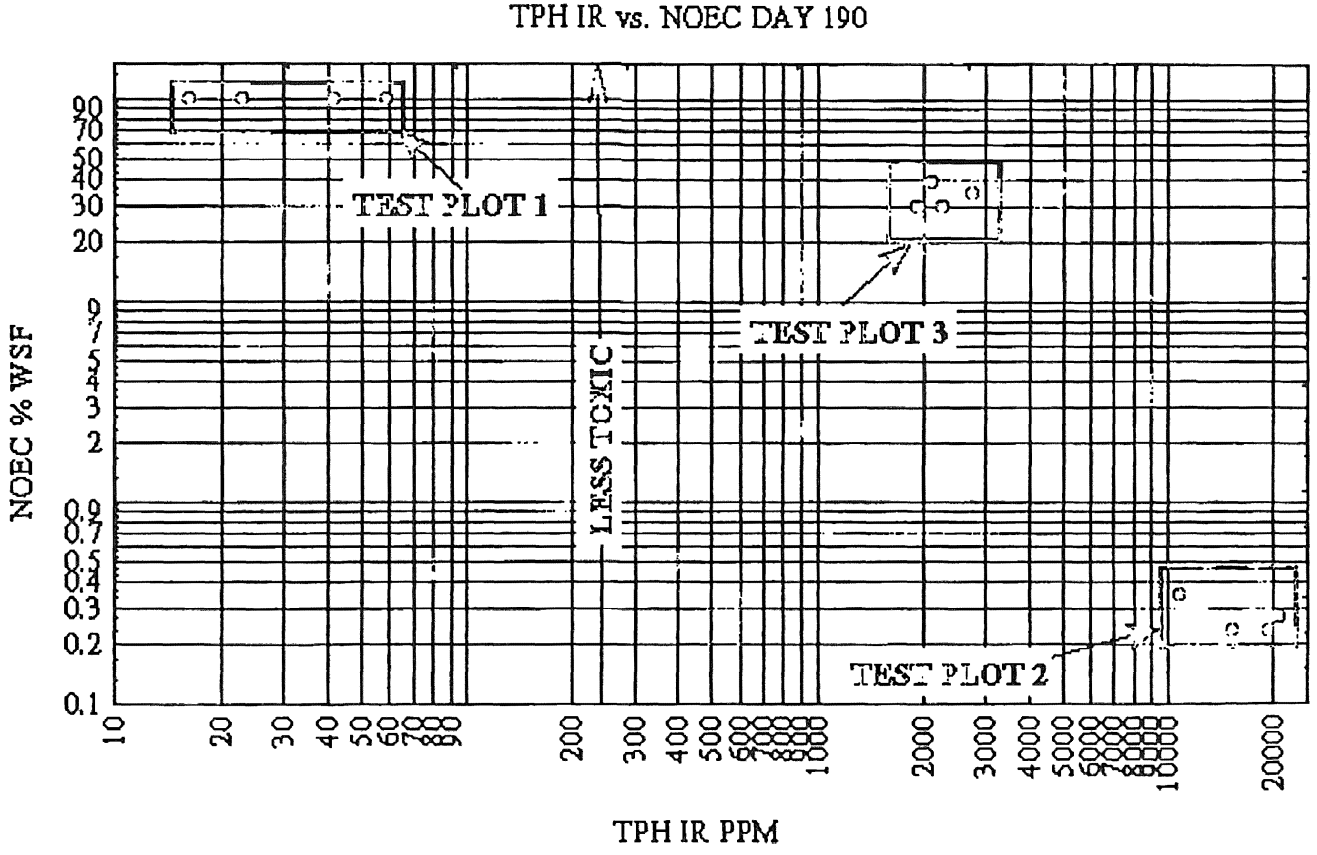


Figure 28. TPH IR vs. NOEC % WSF, Test Plots 1-3, Day 190

TABLE IX

SUMMARY OF RESULTS

TEST SOIL	Okay loam used in study was not contaminated by oilfield waste prior to study as evidenced by: EC < 1 mmhos/cm SAR < 1.0 No Sodium Contamination TPH IR = 17.5 ppm TPH GC < 25 ppm No Oil Contamination	
	Respirometry experiment completed with test soil showed it developed a population of hydrocarbon oxidizing microorganisms in response to crude oil contamination.	
REDUCTION OF CONTAMINANTS	UNAMENDED TEST PLOT	WATER AND NUTRIENT AMENDED TEST PLOT
Benzene	< 0.50 ppm by day 14	< 0.50 ppm by day 14
Toluene	< 0.50 ppm by day 42	< 0.50 ppm by day 28
Ethyl-benzene	< 0.50 ppm by day 56	< 0.50 ppm by day 14
Xylenes	11.64 ppm by day 190	< 0.50 ppm by day 88
TPH IR	Reduced from 23,625 ppm to 17,398 ppm in 190 days. A reduction of 6,227 ppm (26%).	Reduced from 18.871 ppm to 2,210 ppm in 190 days. A reduction of 16,661 ppm (88%).
HYDROCARBON OXIDIZING MICROORGANISM O_2 DEMAND	Soil inoculum collected day 140 of experient developed O_2 demand of 9.0 mg/kg/hr after 93 hours in respirometer.	Soil inoculum collected day 140 of experiment developed O_2 demand of 35.1 mg/kg/hr after 30 hours in respirometer.
MICROTOX® TOXICITY		
EC 50% WSF	22.9% day 14 to 21.8% day 190	31.4% day 14 to 17[illegible]% day 190
% Inhibition by 20% WSF	40% day 14 to 36% day 190	38% day 14 to 10[illegible] day 190
EC 50 by LSPT day 190	.15%	.55%
NOEC of WSF day 190	0.25%	32.2%

CHAPTER V

CONCLUSIONS

The characterization tests of the Okay loam used in this study indicate no previous oil field waste contamination. It can be concluded that the soil has not produced a large population of hydrocarbon oxidizing microorganisms in response to a prior crude oil contamination. The uncontaminated test soil provided an excellent subject for determination of the *in-situ* microorganisms' response to crude oil contamination. Microtox®toxicity measurements of the uncontaminated soil WSF provided a background level to compare with WSF toxicity of crude oil contaminated soil. Measurement of toxicity in uncontaminated soil documented a reduction of the soil's WSF toxicity during the experiment. The loss of plant residues toxic to the Microtox®reagent may be the cause of the observed toxicity reduction.

The respirometry experiment conducted on the test soil contaminated with sterilized Michigan Silurian crude oil demonstrated that a population of hydrocarbon oxidizing microorganisms developed in the soil. Respirometry experiments also determined that the microorganism population developed in nutrient and water amended test soil at day 140 was more efficient in oxidizing hydrocarbons than the microorganism population in the unamended test plot. Optimum growth of hydrocarbon oxidizing microorganisms could be accomplished for a remediation by using parallel respirometry experiments to determine the nutrient requirements of the system.

Qualitative analyses of gas chromatogram patterns showed that hydrocarbons present in the nutrient and water amended test soil experienced alteration consistent with

extensive biodegradation in the first 14 days. Gas chromatograph patterns of the hydrocarbons from the unamended test soil indicated that these hydrocarbons had undergone less degradation than hydrocarbons in the nutrient and water amended test soil. Gas chromatographs that could be used quantitatively to evaluate the detailed progress of bioremediation were not available from the commercial laboratory that performed gas chromatography for this study. Because of the variability in commercial laboratories, it is imperative to make an advance determination of experimental requirements and to select a commercial laboratory that will satisfactorily fulfill these needs.

TPH IR measurements in the unamended test soil recorded a reduction of 6,237 ppm (26%) during the 190 day experiment. TPH IR measurements in the amended test soil recorded a reduction of 16,661 ppm (88%) during the 190 day experiment. The findings of TPH IR measurements, respirometry experiments, and gas chromatograph patterns support the conclusion that oxidation of hydrocarbons progressed more rapidly in the amended test soil because of a more efficient population of hydrocarbon oxidizing microorganisms.

Microtox® measurements of soil acute toxicity during remediation can differentiate among strongly remediated and weakly remediated soils. However, the ability to differentiate between these soils varied among Microtox® protocols and for different data manipulations within a single protocol. It was determined that no single Microtox® basic protocol test effective concentration metric (EC50, EC20, EC10 or EC1) could describe toxicity variations over the range of toxicity generated in this study.

The percentage of Microtox® reagent light inhibition produced at 20% WSF (mean of basic protocol data) provided a consistent metric for comparing toxicity changes during the study and showed a good correlation with remaining TPH IR concentrations in the unamended test plot. The Microtox® Large Sample Solid Phase Test (LSPT) protocol proved to be a labor and supply intensive protocol that does not lend itself to handling many samples at minimum dollar expenditure. Mean EC50 values acquired by LSPT protocol

provided a clear-cut toxicity distinction between uncontaminated test soil, nutrient and moisture amended contaminated test soil and unamended contaminated test soil at day 190 of the study. The Microtox®No Observed Effects Concentration (NOEC) protocol, like the LSPT protocol, proved to be labor and supply intensive. The NOEC protocol also provided a clear-cut toxicity distinction between uncontaminated test soil, nutrient and moisture amended contaminated test soil, and unamended contaminated test soil at day 190 of the study. Because the LSPT measure toxicity by direct contact and does not require a toxicant extraction, it is conceptually and operationally simpler than the NOEC protocol.

The success of soil remediation is, as a rule, judged by the reduction of site-specific contaminants to background contamination levels. The justification for and the goal of any remediation is the reduction of risk to man and the environment. It is concluded that Microtox®acute toxicity tests can monitor changes in toxicity that occur during remediation of petroleum hydrocarbons. Microtox® acute toxicity testing could be used to negotiate site-specific risk levels based on toxocity rather than contaminant concentrations.

BIBLIOGRAPHY

Amoco Corp., 1989, Guidelines for the identification of petroleum hydrocarbons by gas chromatography in groundwater investigations. C. Thompson, ed., Amoco Groundwater Management Section proprietary report, 21 p.

Athey, L. A., J. M. Thomas, W. E. Miller and J. Q. Word, 1989, Evaluation of bioassays for designing sediment cleanup strategies at a wood treatment site. *Environmental Toxicology and Chemistry*, v. 8, p. 223-230.

Atlas, R. M., 1988, Biodegradation of hydrocarbons in the environment. *in* G. S. Omenn, ed., Environmental biotechnology, reducing risks from environmental chemicals through biotechnology, New York, Plenum Press, p. 211-222.

Atlas, R. M., 1991, Bioremediation of fossil fuel contaminated soils. *in* R. E. Hinchee, and R. Olkfenbuttel, eds. *In Situ* bioreclamation, Boston, Butterworth-Heineman, p. 14-32.

Barker, G., 1992, personal communication.

Brouwer, H., T. Murphy and L. McArdle, 1990, A sediment-contact bioassay with *Photobacterium phosphoreum*. *Environmental Toxicology and Chemistry*, v. 9, p. 1353-1358.

Bulich, A. A., 1979, Use of luminescent bacteria for determining toxicity in aquatic environments. *in* L. L. Marking and R. A. Kimerle, eds., Aquatic Toxicology, ASTM STP 667, American Society for Testing and Materials, p. 98-106.

Bulich, A. A., M. W. Greene and D. L. Isenberg, 1981, Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents. *in* D. R. Branson and K. L. Dickson, eds., Aquatic Toxicology and Hazard Assessment: Fourth Conference, ASTM STP 737, American Society for Testing and Materials, p. 338-347.

Cassel and Nielson, 1991, Field capacity and available water capacity. *in* C. A. Black, ed., Methods of Soil Analysis, v. 1, Madison, Wisconsin, American Society of Agronomy, p. 915-918.

Cole, E., D. Bartolina and B. Swafford, 1977, Soil survey of Tulsa County, Oklahoma, United States Department of Agriculture Soil Conservation Service, 140 p.

Corseul, H. X. and W. J. Weber, Jr., 1993, Potential biomass limitations for remediation of subsurface systems. *in* the proceedings of the CoBioReM Conference on Bioremediation of Petroleum Hydrocarbons in Soils and Groundwater, Michigan Oil and Gas Association, Lansing, Michigan.

Dassappa, S. M. and R. C. Loehr, 1991, Toxicity reduction in contaminated soil bioremediation processes. *Water Resources*, v. 25, no. 9, p. 1121-1130.

Desai, S., R. Govind and H. Tabak, 1990, Determination of monod kinetics of toxic compounds by respirometry for structure-biodegradability relationships. American Chemical Society Symposium: Emerging technologies in hazardous waste management, American Chemical Society, chap. 9, p. 142-156.

Deuel, L. E., 1991, Evaluation of limiting constituents suggested for land disposal of exploration and production wastes. API Report #4527, American Petroleum Institute, 45 p.

Donnelly, K. C., K. W. Brown, C. S. Anderson, J. C. Thomas and B. R. Scott, 1991, Bacterial mutagenicity and acute toxicity of solvent and aqueous extracts of soil samples from an abandoned chemical manufacturing site. *Environmental Toxicology and Chemistry*, v. 10, p. 1123-1131.

Fisher, B., 1992, personal communcation.

Gough, M. A. 1989, Characterization of unresolved complex mixtures of hydrocarbons. Doctoral Diss., Polytechnic South West, Department of Environmental Sciences, Drake Circus, Plymouth, U.K., p. 415.

Grady, C. P. L., Jr., J. S. Dang, D. M. Harvey, A. Jobbagy and X.-L. Wang, 1989, Determination of biodegradation kinetics through use of electrolytic respirometry. *Water Science Technology*, v. 21, p. 957-968.

Greene, M. W., A. A. Bulich and S. R. Underwood, 1992, Measurement of soil and sediment toxicity to bioluminescent bacteria when in direct contact for a fixed time period. in proceeding of 65th Annual Conference & Exposition of the Water Environment Federation, New Orleans, Louisiana.

Leahy, G. and R. Colwell, 1990, Microbial degradation of hydrocarbons in the environment. Microbiological Reviews, American Society of Microbiology, p. 305-314.

Matthews, J. E. and L. Hastings, 1987, Evaluation of toxicity procedure for screening treatability potential of waste in soil. *Toxicity Assessment Quarterly*, v. 2, p. 265-281.

McGuinness, S. and B. Barisas, 1991, Acute toxicity measurements on aquatic pollutants using microcalorimetry on tissue-cultured cells. *Environmental Science Technology*, v. 25, p. 1092-1098.

Microbics Corp., 1992, Microtox® update manual. Microbics Corporation, Carlsbad, California.

Munkittrick, K. R. and E. A. Power, 1991, The relative sensitivity of Microtox, Daphnid, Rainbow Trout, and Fathead Minnow acute lethality tests. *Environmental Toxicology and Water Quality Journal*, v. 6, p. 35-62.

Petrocelli, S. R., 1985, Chronic Toxicity tests. *in* Rand, G. M. and S. R. Petrocelli, eds., Fundamentals of Aquatic Toxicology Methods and Applications, New Your, Hemisphere Publishing Corp., chap. 4, p. 96-108.

Raterman, K. T., G. W. Barker and D. Denham, 1993, An assessment of the remediation potential of a native Michigan soil contaminated with fresh Silurian crude oil. *in* the proceedings of the CoBioReM Conference on Bioremediation of Petroleum Hydrocarbons in Soils and Groundwater, Michigan Oil and Gas Association, Lansing, Michigan.

Rosenberg, E. and D. L. Gutnick, 1981, The hydrocarbon-oxidizing bacteria. *in* M. P. Star *et al.*, eds., Prokaryotes, New York, Springer-Verlag, v. 1, chap. 72, p. 903-912.

Tarkepea, M., M. Hanson and B. Samuelsson, 1986, Comparison of the Microtox test with the 96-Hr LC50 test for the Harpacticoid *Nitocra spinipes*. *Ecotoxicology and Environmental Safety*, No. 11, p. 127-143.

USEPA, 1993, Proposed modification of the general permit for the western Gulf of Mexico out continental shelf. Water Management Division, U.S. Environmental Protection Agency Region 6, Dallas, Texas, Permit No. GMG290000, 85 p.

USEPA, 1989, Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd edition. Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-89/011, 249 p.

USEPA, 1985, Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio, EPA-600/4-85/014, 162 p.

Vic, E. A., J. D. Berg, S. Bakke, M. Weideborg, B. S. Nesgard and E. Worum, 1992, Assessment of marine toxicity of offshore chemicals. Report to the Norwegian Oil Industry Association, Oslo, Norway. Aquateam, Norwegian Technology Centre A/S. 115 p.

Wang, X. and R. Bartha, 1990, Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. *Soil Biology and Biochemistry*, V. 22, No. 4, p. 501-505.

Wang, X., X. Yu and R. Bartha, 1990, Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. *Environmental Science Technology*, V. 24, No. 7, p. 1086-1089.

APPENDICES

APPENDIX A

SOIL CHARACTERIZATION TERMS DEFINED

SOIL CHARACTERIZATION TERMS DEFINED

To aid the reader in an understanding of soil characterization terms used the following list of brief definitions is provided:

SP Moisture, %	Saturated Percentage is the moisture content of a saturated paste extract. Approximately equivalent to twice the moisture at field capacity.
SP EC, mmhos/cm	Electrical Conductivity: measured at SP Moisture
Soluble Cations, meq/1	(cation concentration of saturated paste extract in meq/1) X SP/1,000.
SAR	Sodium Adsorption Ratio: calculated from cationic distributions as follows:

$$SAR = \frac{Na, \frac{meq}{1}}{\sqrt{\frac{Ca, \frac{meq}{1} + Mg, \frac{meq}{1}}{2}}}$$

SAR is used in conjunction with EC to evaluate potential hazards associated with sodium and soluble salts. SAR values greater than 15 indicate that exchange sites are occupied with Sodium to a degree which results in a soil without structure or dispersed soil (sodic soil) (Deuel, 1991).

CEC, meq/100 g	Cation Exchange Capacity: the capacity of a soil to absorb positively charged ions.
ESP, %	Exchangeable Sodium Percentage: a measure of the degree to which soil exchange sites are saturated with sodium. Determined by the following calculation: ESP % - ((Exchangeable Sodium meq/1,000 g)/CEC meq/100 g) x 100

Base Saturation, %	The percentage of CEC sites occupied by Calcium, Magnesium, Sodium and Potassium

In fertile soils, the base saturation approaches 80% with the bulk saturation distributed mainly as Calcium and Magnesium.

TOC, ppm	Total Organic Carbon
TPH, ppm	Total Petroleum Hydrocarbons
TPH IR, ppm	Total Petroleum Hydrocarbons by Infrared Spectrometry. This method measures the Freon-113 extractable petroleum hydrocarbons in soil. The stretch of a fixed wavelength IR beam passing through the extract is compared to that of a solution of known concentration to calculate TPH IR in ppm.
TPH GC, ppm	Total Petroleum Hydrocarbons by Gas Chromatograph. In this method the total integrated area under the GC curve of a soil solvent extract less the solvent front is divided by the detector response to carbon per gram of sample to yield the TPH GC.

APPENDIX B

SAMPLE NUMBERS AND COLLECTION COORDINATES

SAMPLE NUMBERS AND COLLECTION COORDINATES

The following is a list of sample numbers and coordinates of sampling points used for remediation monitoring experiment:

Sample numbers explained:

Example:	9-2-1-1
1st number	Sampling Event 9
2nd number	Test Plot Number 2
3rd number	Quadrant Number 1 (C Denotes combination of subsamples from all four quadrants)
4th number	Depth Interval
	1 0 TO 12 inches
	2 12 TO 24 inches
	3 24 TO 36 inches

The following coordinates are in inches measured from the center of sample plot:

Sample Event 1 Date 9-10-92 Samples are designated as 0 (ZERO)

Sample	Coordinates			
0-1-1-1	23,36	14,44	34,25	
0-1-2-1	10,13	10,36	27,03	
0-1-3-1	30,13	37,11	24,22	
0-1-4-1	19,25	35,10	31,22	
0-2-1-1	27,24	06,25	29,16	
0-2-2-1	39,32	36,05	23,31	
0-2-3-1	06,19	04,33	02,13	
0-2-4-1	41,13	15,37	34,27	
0-3-1-1	21,22	16,46	40,33	
0-3-2-1	01,11	08,14	33,27	
0-3-3-1	11,02	38,38	45,34	
0-3-4-1	42,26	30,11	36,37	
	Q1	Q2	Q3	Q4
0-1-C-2	23,36	10,13	30,13	19,25
0-1-C-3	23,36	10,13	30,13	19,25

Sample Event 2 Date 9-12-92

This was a limited sampling to get deep samples not collected at event 1.

	Q1	Q2	Q3	Q4
2-2-C-2	04,04	22,29	53,24	43,39

2-2-C-3	04,04	22,29	53,24	43,39

Sample Event 3 Date 9-24-92

3-2-1-1	28,31	19.07	34,41	
3-2-2-1	19,42	40,16	27,28	
3-2-3-1	17,33	26,20	06,43	
3-2-4-1	45,29	22,35	30,48	
	Q1	Q2	Q3	Q4
3-2-C-2	08,31	19,42	17,33	45,29
3-2-C-3	08,31	19,42	17,33	45,29

3-3-1-1	07,36	21,12	01,04	
3-3-2-1	22,19	14,48	35,46	
3-3-3-1	08,21	49,33	24,15	
3-3-4-1	42,05	21,27	17,35	
	Q1	Q2	Q3	Q4
3-3-C-2	07,36	22,19	08,21	42,05
3-3-C-3	07,36	22,19	08,21	42,05

Sample Event 4 Date 10-08-92

Note: Due to sample volumes required to satisfy laboratory needs the number of sampling points per quadrant was increased from three to four.

4-2-1-1	49,38	26,01	33,12	24,36
4-2-2-1	38,26	08,31	46,31	16,24
4-2-3-1	14,28	28,02	39,11	30,11
4-2-4-1	00,13	44,17	33,39	25,22
	Q1	Q2	Q3	Q4
4-2-C-2	49,38	38,26	14,28	00,13
4-2-C-3	49,38	38,26	14,28	00,13
4-3-1-1	02,35	37,38	17,24	21,02
4-3-2-1	06,33	31,13	36,01	44,24
4-3-3-1	43,16	37,11	15,31	35,48
4-3-4-1	09,02	19,30	42,06	26,35
	Q1	Q2	Q3	Q4
4-3-C-2	02,35	06,33	43,16	09,02
4-3-C-3	02,35	06,33	43,16	09,02

Sample Event 5 Date 10/22/92

5-1-1-1	44,32	20,21	00,19	40,18
5-1-2-1	17,14	46,28	07,20	25,14
5-1-3-1	44,29	30,31	26,05	21,42
5-1-4-1	44,04	04,33	22,46	07,22
	Q1	Q2	Q3	Q4
5-1-C-2	44,32	17,14	44,29	44,04
5-1-C-3	44,32	17,14	44,29	44,04

5-2-1-1	16,38	17,17	21,44	08,41
5-2-2-1	43,28	10,28	23,43	11,22
5-2-3-1	32,01	01,34	21,08	19,10
5-2-4-1	16,04	00,19	03,19	39,01
	Q1	Q2	Q3	Q4
5-2-C-2	16,38	43,28	32,01	16,04
5-2-C-3	16,38	43,28	32,01	16,04
5-3-1-1	18,18	48,00	23,45	48,12
5-3-2-1	36,20	25,33	03,15	30,15
5-3-3-1	20,14	05,01	18,46	20,10
5-3-4-1	27,24	46,27	33,42	01,44
	Q1	Q2	Q3	Q4
5-3-C-2	18,18	36,20	20,14	27,24
5-3-C-3	18,18	36,20	20,14	27,24

Sample Event 6 Date 11/23/92

6-1-1-1	15,49	19,14	02,12	09,17
6-1-2-1	20,06	01,29	27,06	36,18
6-1-3-1	21,34	41,19	39,43	10,11
6-1-4-1	42,41	19,07	24,34	31,38
	Q1	Q2	Q3	Q4
6-1-C-2	15,49	20,06	21,34	42,41
6-1-C-3	15,49	20,06	21,34	42,41
6-2-1-1	30,49	01,13	26,35	39,31
6-2-2-1	03,22	37,11	43,43	04,11
6-2-3-1	16,04	40,19	39,01	17,11
6-2-4-1	32,01	01,34	21,08	19,10
	Q1	Q2	Q3	Q4
6-2-C-2	30,49	03,22	16,04	32,01
6-2-C-3	30,49	03,22	16,04	32,01
6-3-1-1	16,29	38,45	01,11	32,11
6-3-2-1	38,01	48,21	30,47	38,06
6-3-3-1	20,10	23,30	00,23	25,18
6-3-4-1	46,27	33,42	01,44	04,38
	Q1	Q2	Q3	Q4
6-3-C-2	16,29	38,01	20,10	46,27
6-3-C-3	16,29	38,01	20,10	46,27

Sample Event 7 Date 12/23/92

7-1-1-1	39,12	11,38	26,26	23,17
7-1-2-1	04,07	39,19	44,23	19,22
7-1-3-1	48,47	07,06	14,26	06,42
7-1-4-1	21,49	47,12	16,48	13,39

	Q1		Q2	Q3	Q4
7-1-C-2	39,12		04,07	48,47	21,49
7-1-C-3	39,12		04,07	48,47	21,49
7-2-1-1	39,08		23,05	18,33	17,40
7-2-2-1	19,02		28,08	21,10	17,40
7-2-3-1	27,31		04,36	01,29	04,18
7-2-4-1	18,35		18,30	25,40	46,25
	Q1		Q2	Q3	Q4
7-2-C-2	39,08		19,02	27,31	18,35
7-2-C-3	39,08		19,02	27,31	18,35
7-3-1-1	12,01		29,23	42,10	27,26
7-3-2-1	11,33		27,08	31,17	32,44
7-3-3-1	31,01		48,35	48,21	39,01
7-3-4-1	04,38		51,46	39,01	48,21
	Q1		Q2	Q3	Q4
7-3-C-2	12,01		11,33	31,01	04,38
7-3-C-3	12,01		11,33	31,01	04,38
Sample Event 8 Date 01/22/93					
8-1-1-1	49,49		11,24	31,15	11,42
8-1-2-1	11,05		25,51	05,51	39,38
8-1-3-1	32,22		40,27	27,13	25,10
8-1-4-1	13,08		15,19	25,01	06,18
	Q1		Q2	Q3	Q4
8-1-C-2	49,49		11,05	32,22	13,08
8-1-C-3	49,49		11,05	32,22	13,08
8-2-1-1	30,10		07,29	26,10	17,28
8-2-2-1	22,04		10,40	36,49	11,38
8-2-3-1	43,37		49,09	04,51	28,40
8-2-4-1	04,19		06,12	23,03	18,16
	Q1		Q2	Q3	Q4
8-2-C-2	30.10		22,04	43,37	04,19
8-2-C-3	30.10		22,04	43,37	04,19
8-3-1-1	44,31		01,28	10,25	43,28
8-3-2-1	45,40	38,17	05,50	12,25	
8-3-3-1	23,26		10,17	20,02	05,19
8-3-4-1	22,02		21,50	50,27	05,34
	Q1		Q2	Q3	Q4
8-3-C-2	44,31		45,40	23,26	22,02

8-3-C-3	44,31	45,40	23,26	22,02

Sample Event 9 Date 03/5/93

9-1-1-1	02,30	13,24	44,12	08,36
9-1-2-1	42,13	50,37	21,30	13,24
9-1-3-1	36,52	36,37	15,35	36,49
9-1-4-1	51,35	00,40	04,37	16,02
	Q1	Q2	Q3	Q4
9-1-C-2	02,30	42,13	36,52	51,35
9-1-C-3	02,30	42,13	36,52	51,35
9-2-1-1	00,19	48,27	31,25	38,01
9-2-3-1	16,44	46,17	14,21	35,45
9-2-4-1	39,11	31,30	20,12	10,27
	Q1	Q2	Q3	Q4
9-2-C-2	00,19	51,27	16,44	39,11
9-2-C-3	00,19	51,27	16,44	39,11
9-3-1-1	06,06	30,02	45,36	16,25
9-3-2-1	11,01	35,50	05,23	19,25
9-3-3-1	05,44	39,37	49,14	39,23
9-3-4-1	08,14	05,37	26,13	05,19
	Q1	Q2	Q3	Q4
9-3-C-2	06,06	11,01	05,44	08,14
9-3-C-3	06,06	11,01	05,44	08,14

Figures 29-31 are maps of sample hole locations for test plots Nos. 1-3 respectively.

Test Plot No. 1

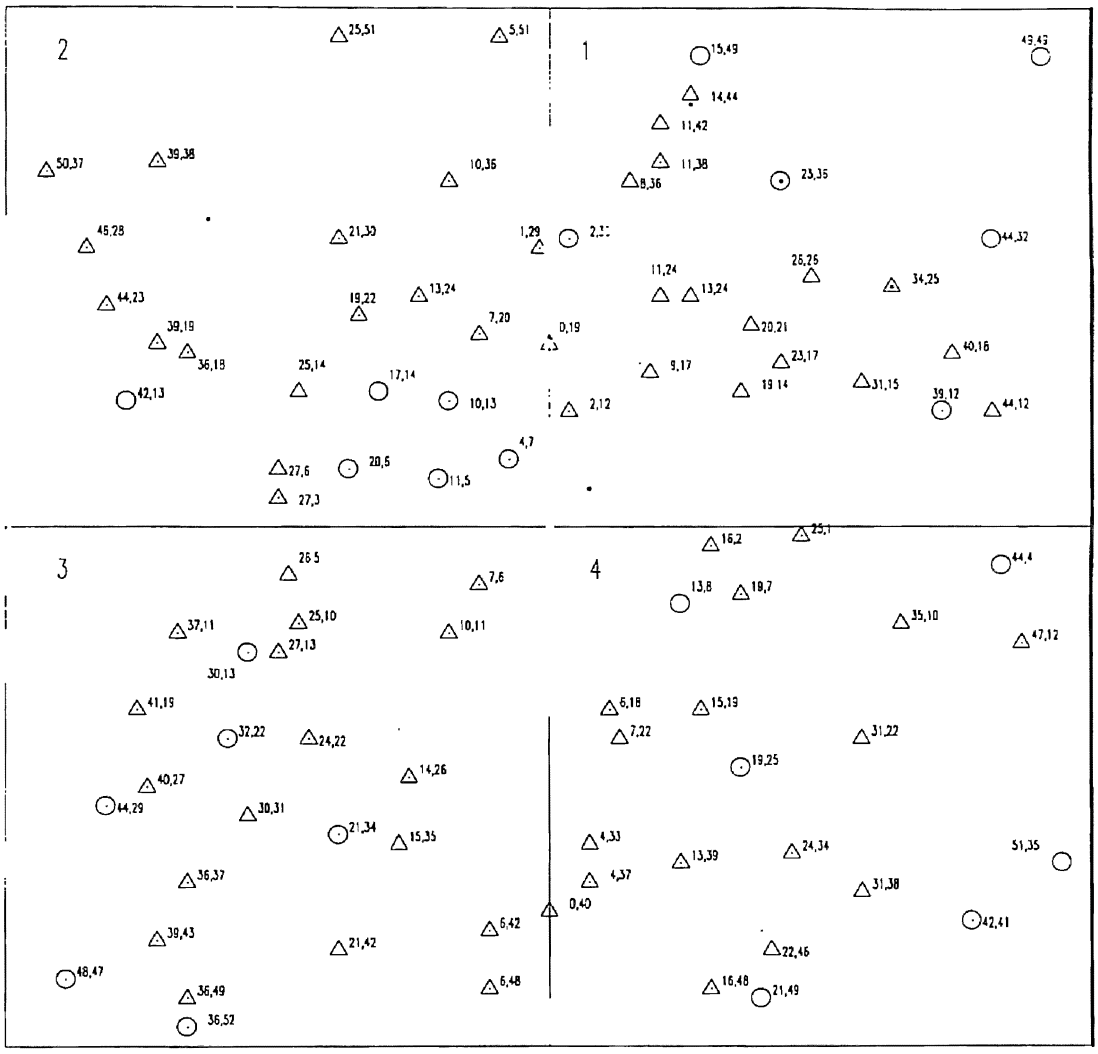


Figure 29. Test Plot No. 1 Sample Hole Locations

Test Plot No. 2

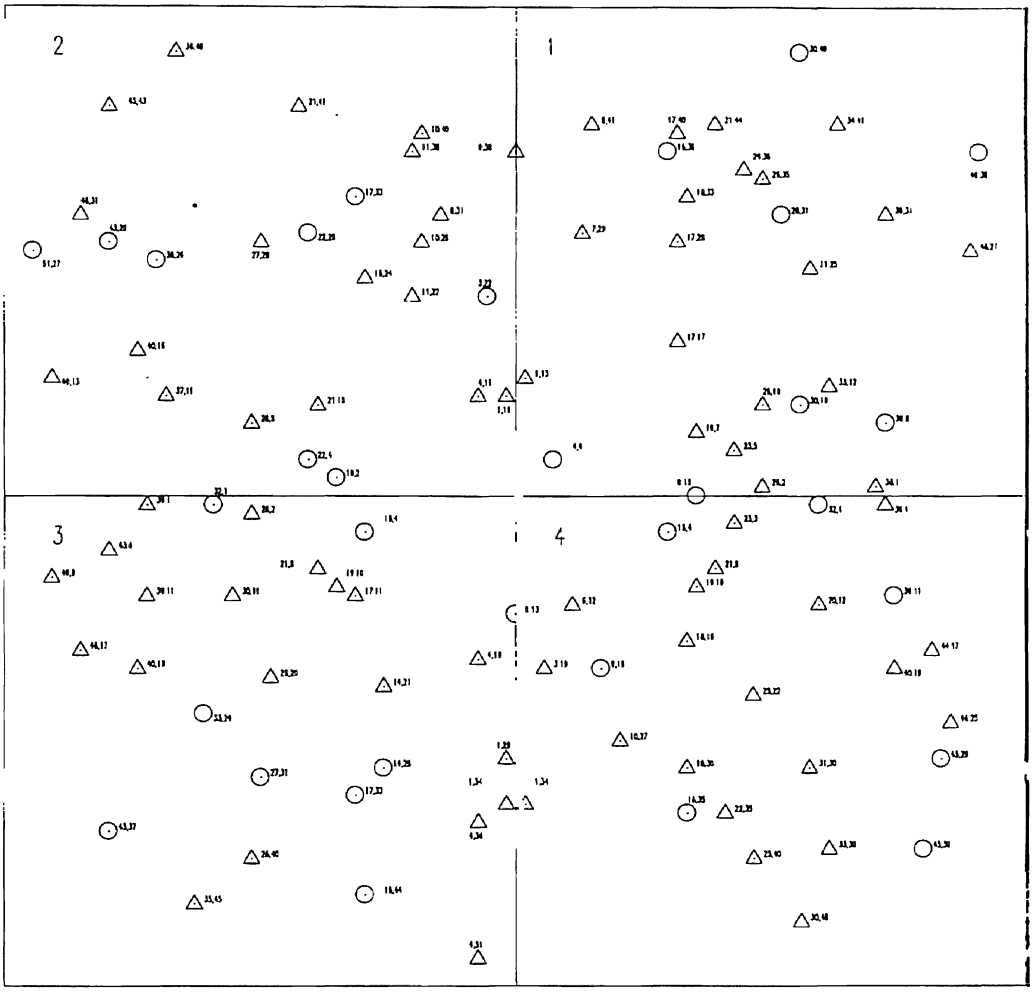
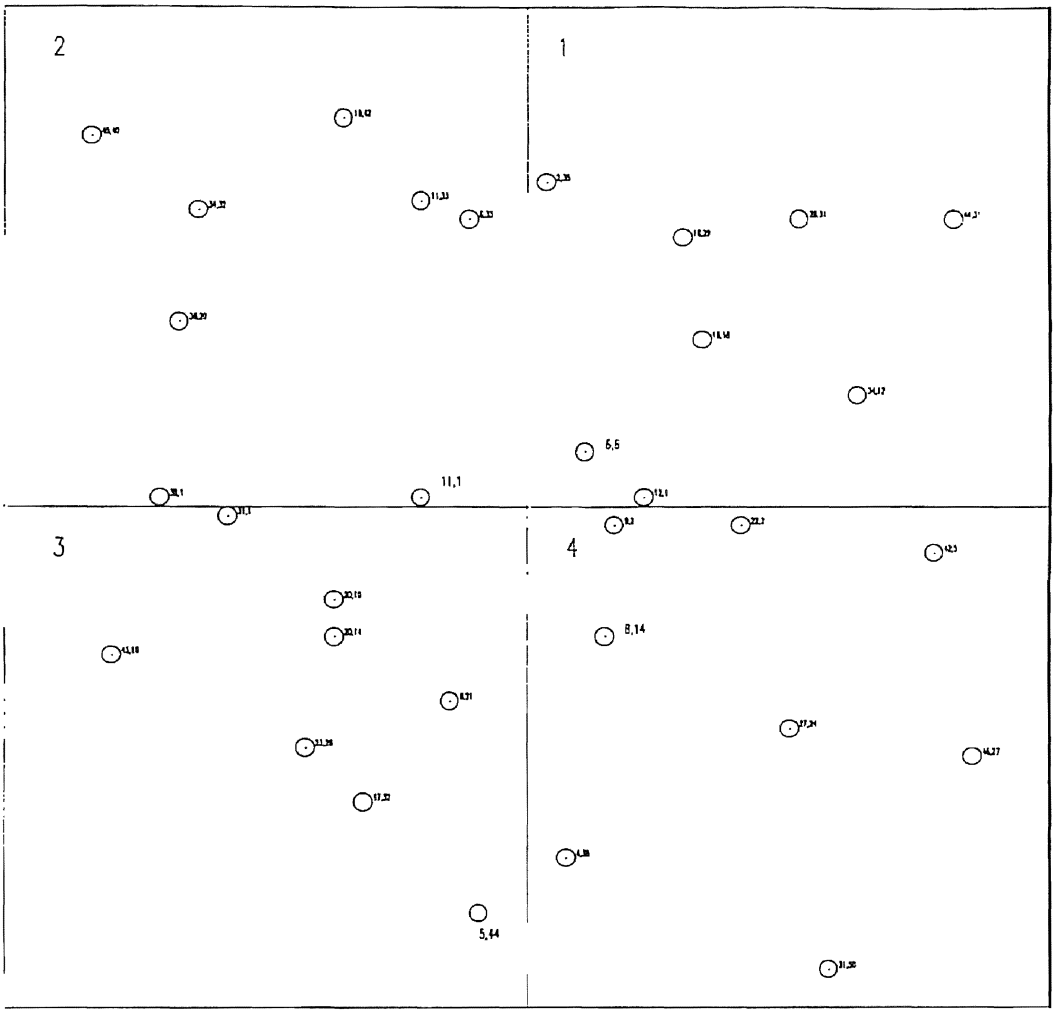


Figure 30. Test Plot No. 2 Sample Hole Locations

Test Plot No. 3



DEEP SAMPLE HOLES

Test Plot #3 was tilled to a depth of 12 inches each week. Only concerned with sampling same position in deep holes.

Figure 31. Test Plot No. 3 Sample Hole Locations

APPENDIX C

TPH IR, TPH GC AND BTEX MEASUREMENTS OF REMEDIATION SAMPLES

TABLE X

TPH IR, TPH GC AND BTEX MEASUREMENTS OF REMEDIATION SAMPLES

SAMPLE #	TPH IR PPM	TPH GC PPM	BENZENE PPM	TOLUENE PPM	ETHYL BENZENE PPM	XYLENES PPM
0-1-1-1	44	119	<0.50	<0.50	<0.50	<0.50
0-1-2-1	79	92	<0.50	<0.50	<0.50	<0.50
0-1-3-1	52	96	<0.50	1.47	<0.50	<0.50
0-1-4-1	65	423	<0.50	0.73	0.58	1.83
0-2-1-1	15,574	15,945	<0.50	5.17	18.13	99.9
0-2-2-1	58,795	31,219	<0.50	52.28	55.91	152.5
0-2-3-1	28,890	32,124	<0.50	27	45.91	145.1
0-2-4-1	18,381	21,798	<0.50	5.84	16.11	91.84
2-2-C-2	5,976	2,815	<0.50	1.99	2.28	12.14
2-2-C-3	3,926	4,212	<0.50	<0.50	0.76	6.87
0-3-1-1	16,541	7,766	<0.50	9.36	9.26	48.9
0-3-2-1	23,515	11,866	<0.50	7.38	<0.50	90.45
0-3-3-1	13,112	6,645	<0.50	1.93	<0.50	91.05
0-3-4-1	21,201	15,672	<0.50	3.07	<0.50	29.47
2-3-C-2	5,562	2,787	<0.50	1.27	<0.50	14.1
2-3-C-3	3,941	3,786	<0.50	1.65	<0.50	10.13
3-2-1-1	25,954	19,060	<0.50	0.52	<0.50	23.7
3-2-2-1	24,699	22,714	<0.50	0.58	<0.50	29.8
3-2-3-1	35,871	21,318	<0.50	1.67	<0.50	33.5
3-2-4-1	41,612	24,504	<0.50	1.8	<0.50	27.3
3-3-1-1	25,667	6,921	<0.50	0.7	<0.50	14.1
3-3-2-1	19,603	3,715	<0.50	<0.50	<0.50	8.92
3-3-3-1	25,868	4,116	<0.50	<0.50	<0.50	3.42
3-3-4-1	16,582	7,153	<0.50	<0.50	<0.50	3.34
4-2-1-1	16,918	20,986	<0.50	<0.50	1.89	23.32
4-2-2-1	20,088	28,404	<0.50	<0.50	1	60.25
4-2-3-1	27,168	26,545	<0.50	<0.50	4.34	55.25
4-2-4-1	29,055	26,601	<0.50	0.59	6.72	43.25
4-2-C-2	4,390	7,804	<0.50	<0.50	<0.50	1.54
4-3-1-1	19,005	17,648	4.05	0.52	<0.50	8.66
4-3-2-1	18,791	12,674	<0.50	<0.50	<0.50	7.77
4-3-3-1	8,142	6,813	<0.50	0.63	<0.50	5.17
4-3-4-1	15,492	12,485	<0.50	<0.50	<0.50	1.25
4-3-C-2	5,147	1,515	<0.50	<0.50	<0.50	<0.50
4-3-C-3	3,090	1,101	<0.50	<0.50	<0.50	0.64
5-1-1-1	16	<25	<0.50	<0.50	<0.50	<0.50
5-1-2-1	12	<25	<0.50	<0.50	<0.50	<0.50
5-1-3-1	13	<25	<0.50	<0.50	<0.50	<0.50
5-1-4-1	8	<25	<0.50	<0.50	<0.50	<0.50
5-1-C-2	19	<25	<0.50	<0.50	<0.50	<0.50
5-1-C-3	21	<25	<0.50	0.65	<0.50	16.5

TABLE X

TPH IR, TPH GC AND BTEX MEASUREMENTS OF REMEDIATION SAMPLES (Continued)

SAMPLE #	TPH IR PPM	TPH GC PPM	BENZENE PPM	TOLUENE PPM	ETHYL BENZENE PPM	XYLENES PPM
5-2-1-1	13,286	14,770	<0.50	<0.50	<0.50	1.02
5-2-2-1	17,991	16,808	<0.50	<0.50	0.61	57.2
5-2-3-1	24,247	24,911	<0.50	<0.50	<0.50	54.1
5-2-4-1	19,855	24,911	<0.50	0.96	<0.50	46.2
5-2-C-2	2,062	2,897	<0.50	<0.50	<0.50	<0.50
5-2-C-3	3,585	4,419	<0.50	<0.50	<0.50	3.8
5-3-1-1	3,403	7,770	<0.50	0.89	0.94	0.71
5-3-2-1	11,595	6,864	<0.50	1.04	<0.50	1.76
5-3-3-1	8,145	4,791	<0.50	0.82	<0.50	2.19
5-3-4-1	7,729	4,796	<0.50	0.79	<0.50	0.78
5-3-C-2	546	3,631	<0.50	<0.50	<0.50	<0.50
5-3-C-3	1,963	784	<0.50	<0.50	<0.50	<0.50
6-1-1-1	16	<25	<0.50	<0.50	<0.50	<0.50
6-1-2-1	26	<25	<0.50	<0.50	<0.50	<0.50
6-1-3-1	17	<25	<0.50	<0.50	<0.50	<0.50
6-1-4-1	28	<25	<0.50	1.15	<0.50	<0.50
6-1-C-2	12	<25	<0.50	<0.50	<0.50	0.69
6-1-C-3	10	<25	<0.50	1.2	<0.50	<0.50
6-2-1-1	9,392	6,778	<0.50	<0.50	<0.50	4.03
6-2-2-1	10,850	11,180	<0.50	1.49	<0.50	2.9
6-2-3-1	15,581	16,420	<0.50	<0.50	<0.50	11.19
6-2-4-1	19,567	16,941	<0.50	<0.50	<0.50	8.55
6-2-C-2	9,471	7,440	<0.50	1.36	<0.50	4.35
6-2-C-3	4,677	6,222	1.8	2.96	<0.50	6.12
6-3-2-1	15,259	6,382	<0.50	<0.50	<0.50	<0.50
6-3-3-1	8,999	3,749	<0.50	1.81	<0.50	<0.50
6-3-4-1	9,036	3,700	<0.50	<0.50	<0.50	<0.50
6-3-C-2	3,560	1,173	<0.50	<0.50	<0.50	<0.50
6-3-C-3	836	1,077	<0.50	<0.50	<0.50	<0.50
7-1-1-1	25	<25	<0.50	<0.50	<0.50	0.69
7-1-2-1	29	<25	<0.50	<0.50	<0.50	0,81
7-1-3-1	8	<25	<0.50	<0.50	<0.50	0.58
7-1-4-1	21	<25	<0.50	<0.50	<0.50	<0.50
7-1-C-2	19	<25	<0.50	<0.50	<0.50	<0.50
7-1-C-3	18	<25	<0.50	<0.50	<0.50	<0.50
7-2-1-1	45,612	23,513	<0.50	<0.50	<0.50	4.95
7-2-2-1	47,836	22,722	0.63	<0.50	<0.50	35.76
7-2-3-1	57,112	28,858	0.78	<0.50	<0.50	60.74
7-2-4-1	49,035	15,087	<0.50	<0.50	<0.50	27.26
7-2-C-2	2,773	1,,432	<0.50	<0.50	<0.50	3.17
7-2-C-3	3,395	2,481	<0.50	<0.50	<0.50	2.41
7-3-1-1	8,176	2,686	<0.50	<0.50	<0.50	<0.50
7-3-2-1	11,765	2,299	<0.50	<0.50	<0.50	<0.50
7-3-3-1	10,129	1,086	<0.50	<0.50	<0.50	<0.50
7-3-4-1	7,864	1,434	<0.50	<0.50	<0.50	<0.50
7-3-C-2	1,109	470	<0.50	<0.50	<0.50	<0.50
7-3-C-3	1,039	139	<0.50	<0.50	<0.50	<0.50
8-1-1-1	45	<25	<0.50	<0.50	<0.50	<0.50
8-1-2-1	40	<25	<0.50	<0.50	<0.50	<0.50

TABLE X

TPH IR, TPH GC AND BTEX MEASUREMENTS OF REMEDIATION SAMPLES (Continued)

SAMPLE #	TPH IR PPM	TPH GC PPM	BENZENE PPM	TOLUENE PPM	ETHYL BENZENE PPM	XYLENES PPM
8-1-3-1	31	<25	<0.50	<0.50	<0.50	<0.50
8-1-4-1	34	40	<0.50	<0.50	<0.50	<0.50
8-1-C-2	56	<25	<0.50	<0.50	<0.50	<0.50
8-1-C-3	39	<25	<0.50	<0.50	<0.50	<0.50
8-2-1-1	24,878	13,194	<0.50	<0.50	<0.50	14.71
8-2-2-1	15,667	7,146	<0.50	<0.50	<0.50	15.12
8-2-3-1	27,587	12,767	<0.50	<0.50	<0.50	31.84
8-2-4-1	34,462	16,956	<0.50	0.86	0.92	19.75
8-2-C-2	3,910	1,950	<0.50	<0.50	<0.50	4.25
8-2-C-3	2,521	1,041	<0.50	<0.50	<0.50	2.1
8-3-1-1	8,436	2,508	<0.50	<0.50	<0.50	<0.50
8-3-2-1	9,067	1,183	<0.50	<0.50	<0.50	<0.50
8-3-3-1	7,599	826	<0.50	<0.50	<0.50	<0.50
8-3-4-1	6,885	877	<0.50	<0.50	<0.50	<0.50
8-3-C-2	2,741	729	<0.50	0.52	<0.50	<0.50
8-3-C-3	757	59	<0.50	<0.50	<0.50	<0.50
9-1-1-1	59	<25	<0.50	<0.50	<0.50	<0.50
9-1-2-1	16	<25	<0.50	0.59	<0.50	1.43
9-1-3-1	23	<25	<0.50	<0.50	<0.50	0.7
9-1-4-1	42	<25	<0.50	<0.50	<0.50	0.7
9-1-C-2	25	<25	<0.50	<0.50	<0.50	<0.50
9-1-C-3	17	<25	<0.50	<0.50	<0.50	<0.50
9-2-1-1	10,824	6,129	0.67	1.06	0.85	4.17
9-2-2-1	20,868	12,167	<0.50	<0.50	<0.50	14.6
9-2-3-1	19,392	10,485	<0.50	<0.50	<0.50	22.63
9-2-4-1	15,404	7,732	<0.50	<0.50	<0.50	8.67
9-2-C-2	7,395	5,088	<0.50	<0.50	<0.50	3.35
9-2-C-3	7,677	4,701	<0.50	<0.50	<0.50	2.2
9-3-1-1	2,294	1,247	<0.50	<0.50	<0.50	<0.50
9-3-2-1	1,937	1,120	<0.50	<0.50	<0.50	<0.50
9-3-3-1	2,781	472	<0.50	<0.50	<0.50	<0.50
9-3-4-1	2,126	567	<0.50	<0.50	<0.50	<0.50
9-3-C-2	183	<25	<0.50	<0.50	<0.50	<0.50
9-3-C-3	105	<25	<0.50	<0.50	<0.50	<0.50

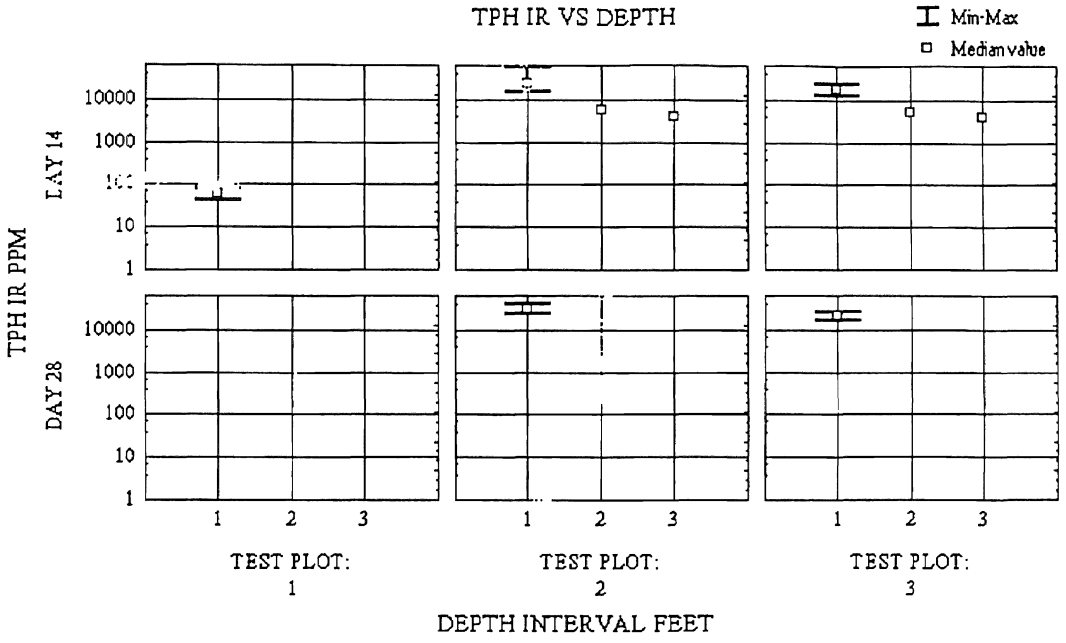


Figure 32. TPH IR PPM VS. Depth, Day 14 and Day 28

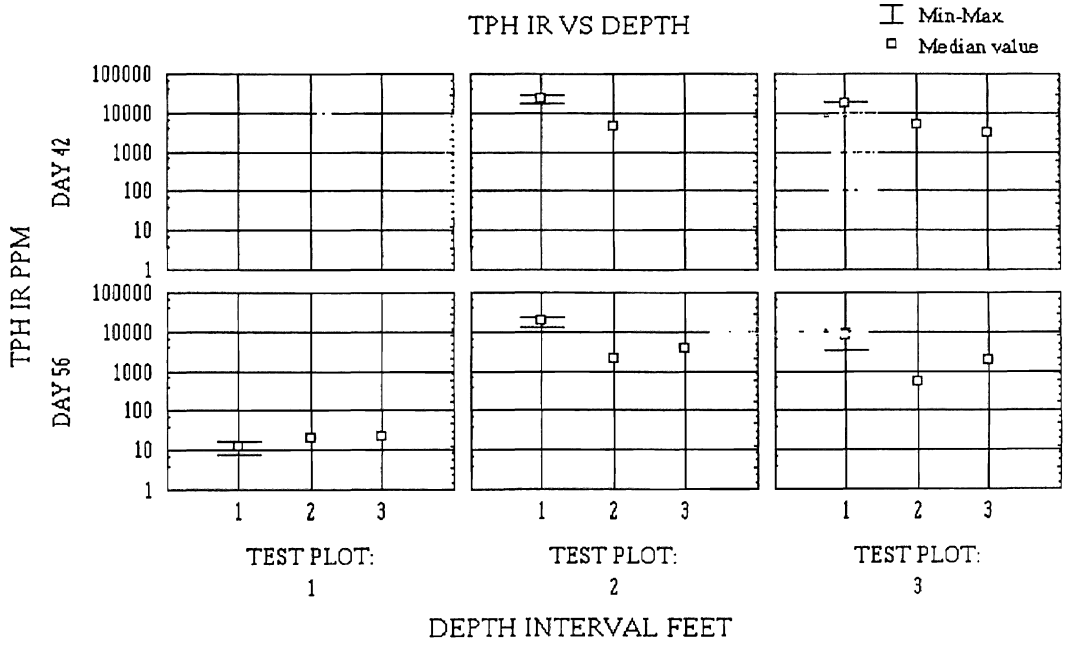


Figure 33. TPH IR ppm VS. Depth, Day 42 and Day 56

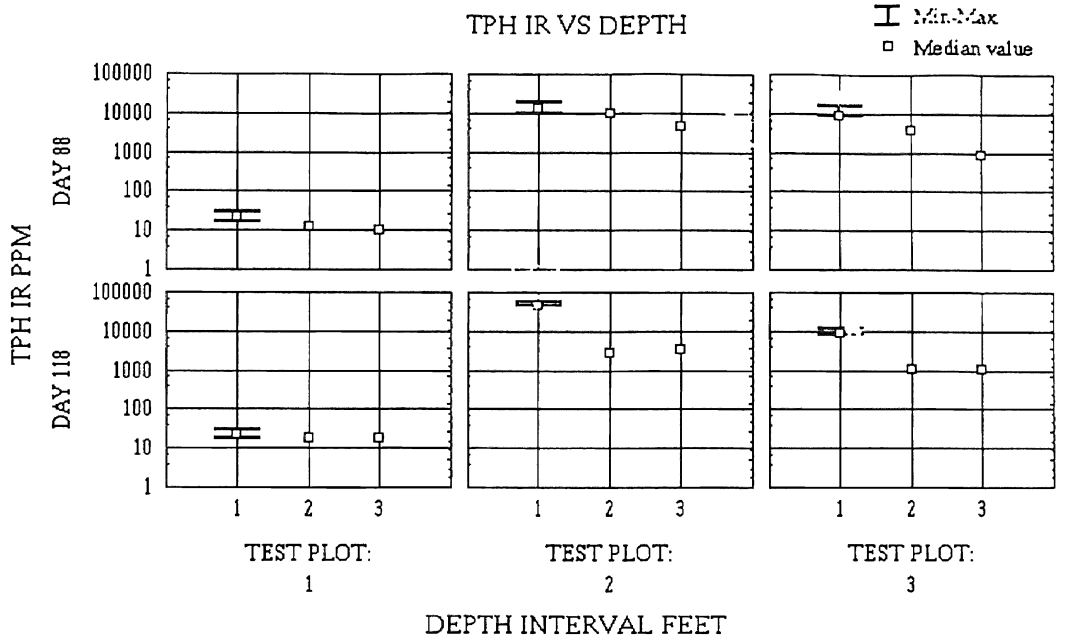


Figure 34. TPH IR ppm VS. Depth, Day 88 and Day 118

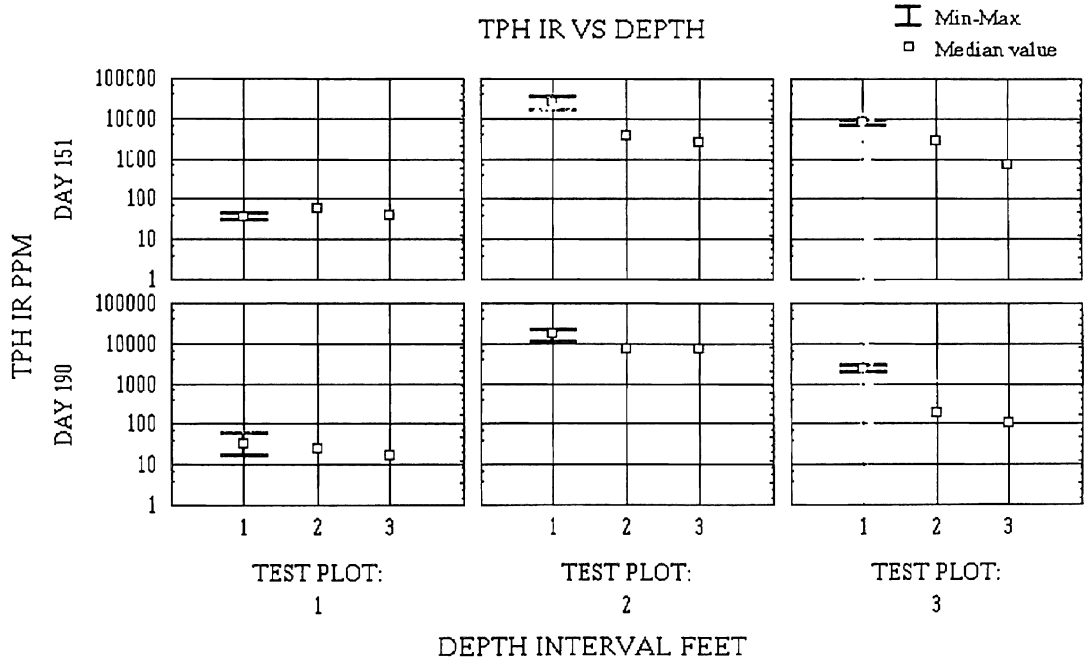


Figure 35. TPH IR ppm VS. Depth, Day 151 and Day 190

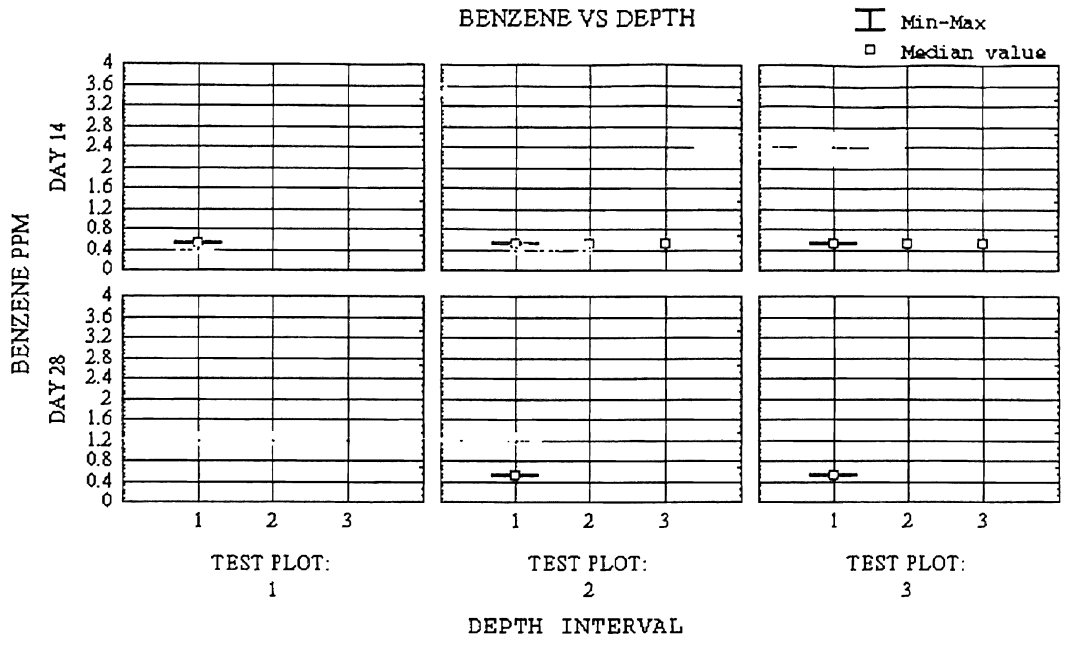


Figure 36. Benzene Concentration vs. Depth, Days 14 and 28

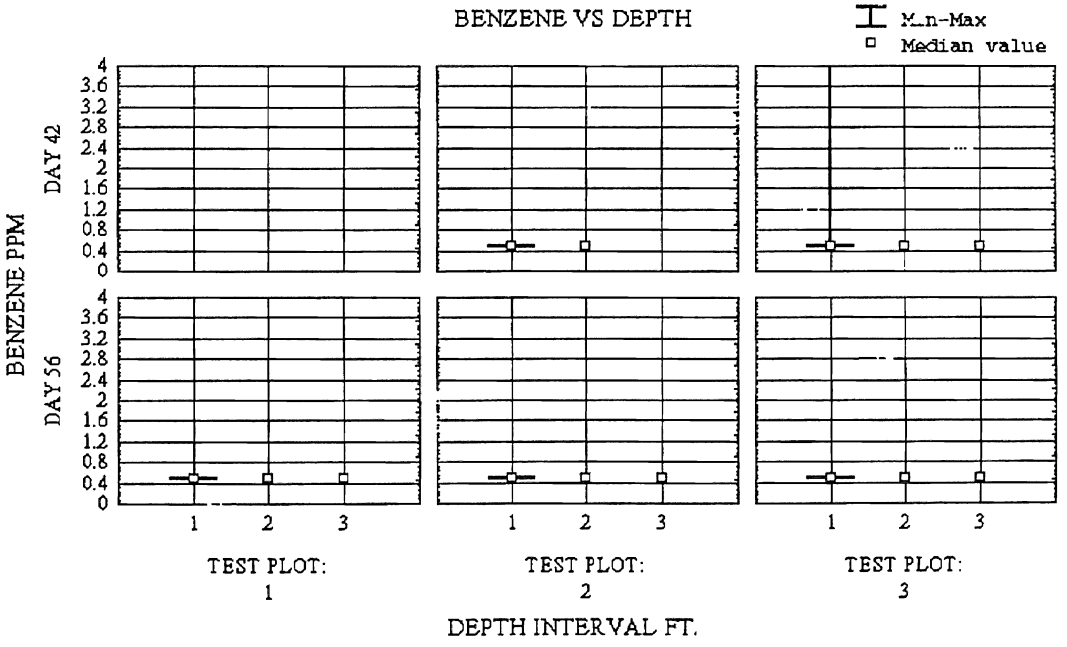


Figure 37. Benzene Concentration vs. Depth, Days 42 and 56

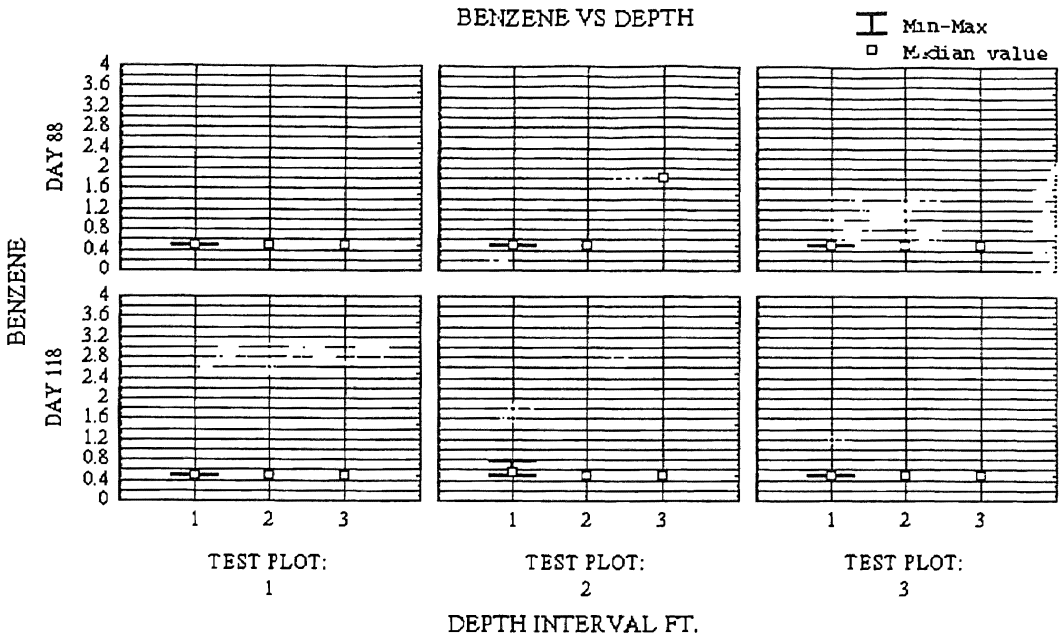


Figure 38. Benzene vs. Depth, Days 88 and 118

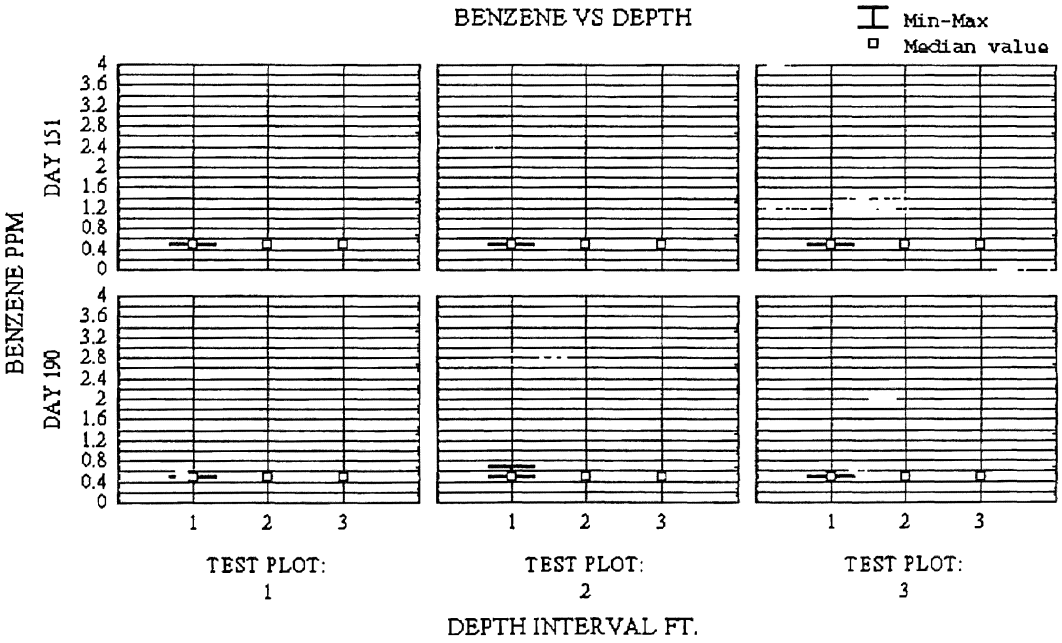


Figure 39. Benzene vs. Depth, Days 151 and 190

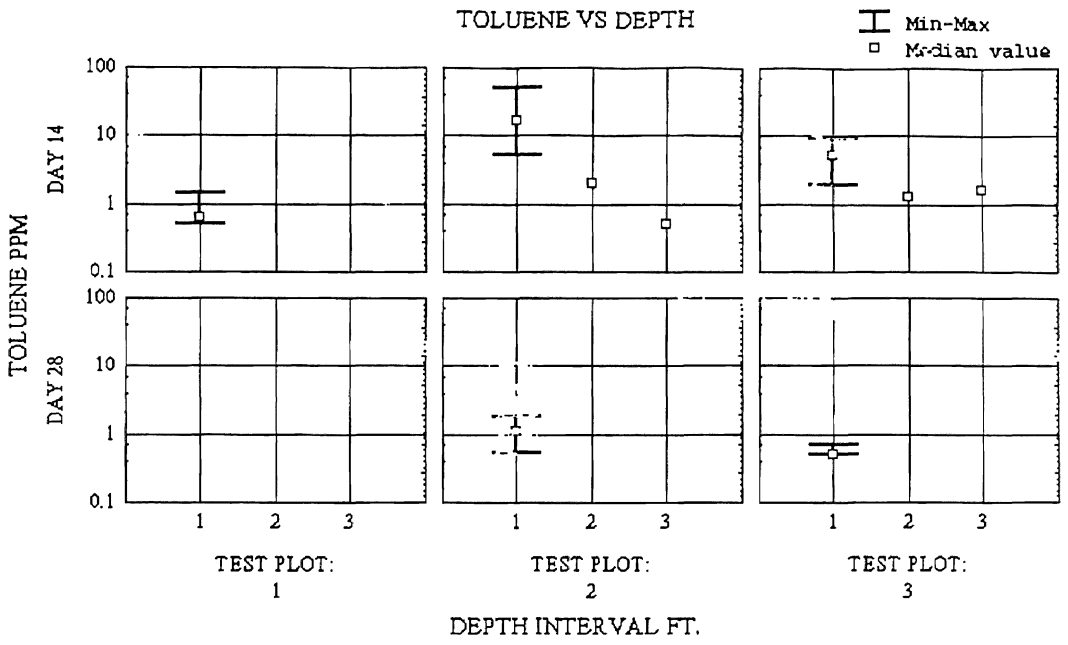


Figure 40. Toluene vs. Depth, Days 14 and 28

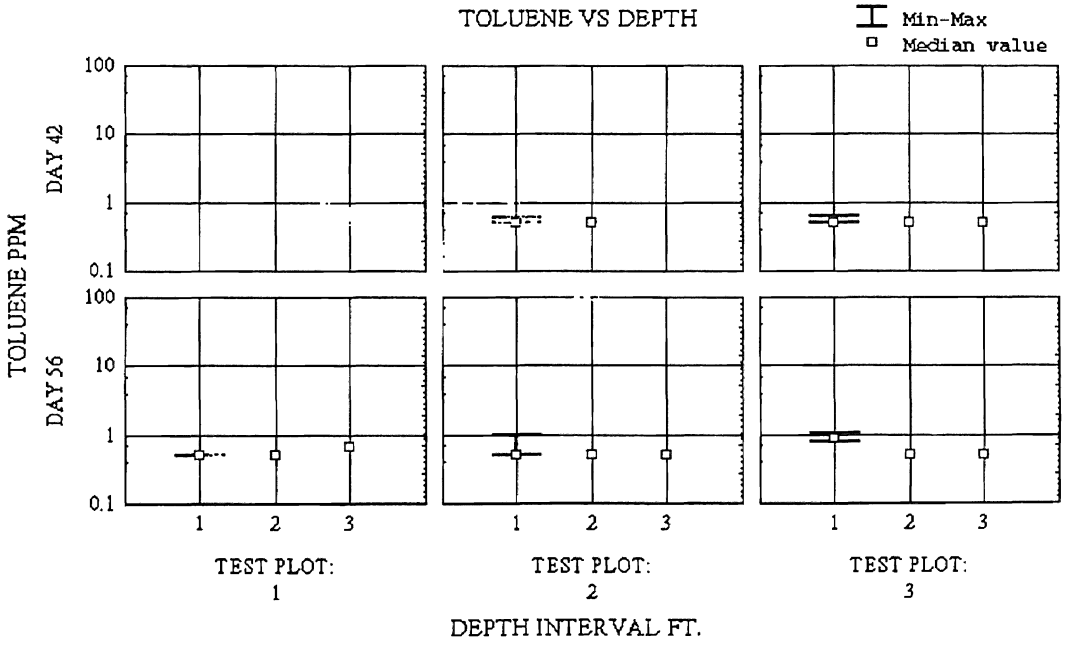


Figure 41. Toluene vs. Depth, Days 42 and 56

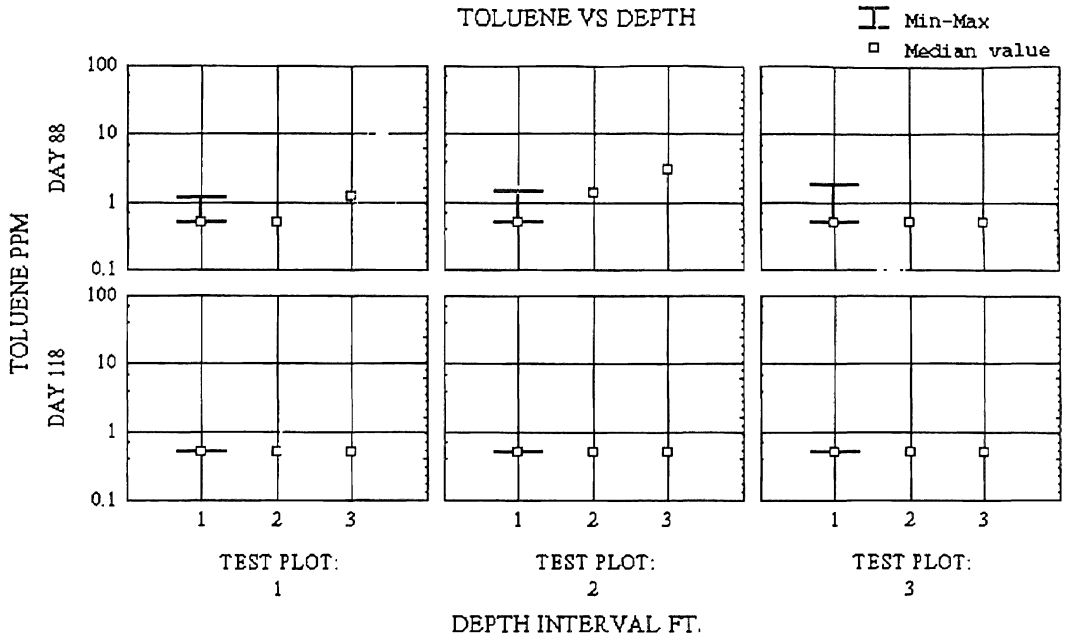


Figure 42. Toluene vs. Depth, Days 88 and 118

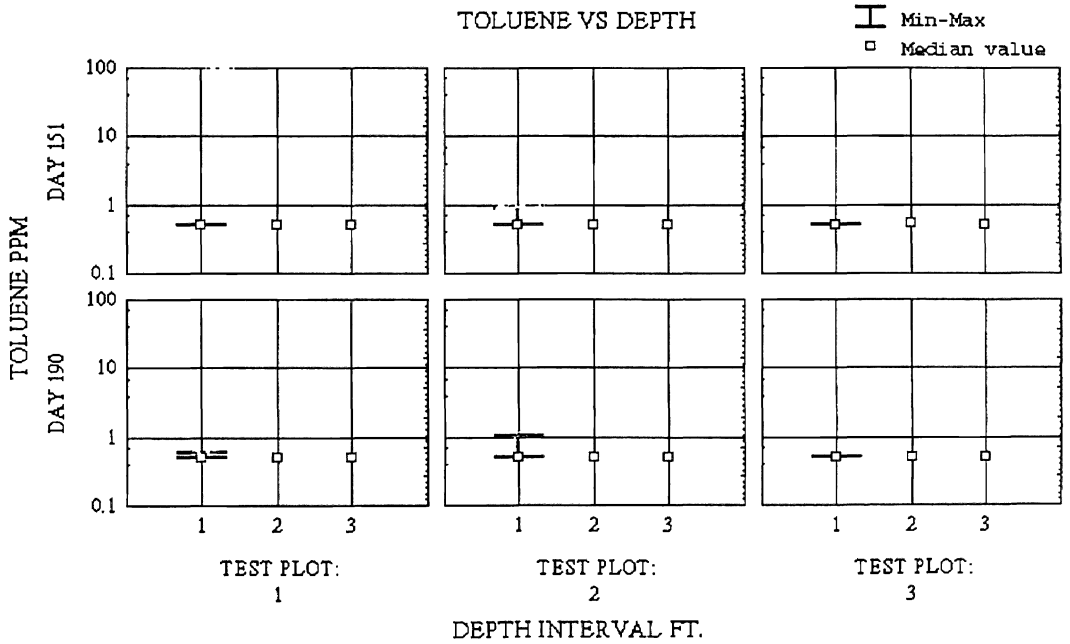


Figure 43. Toluene vs. Depth, Days 151 and 190

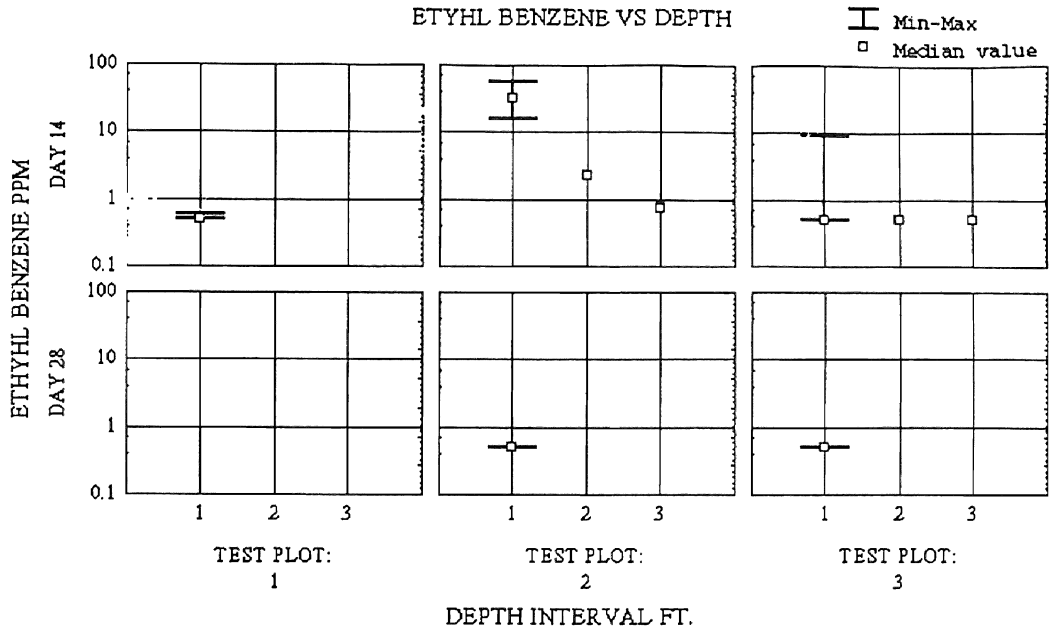


Figure 44. Ethyl Benzene vs. Depth, Days 14 and 28

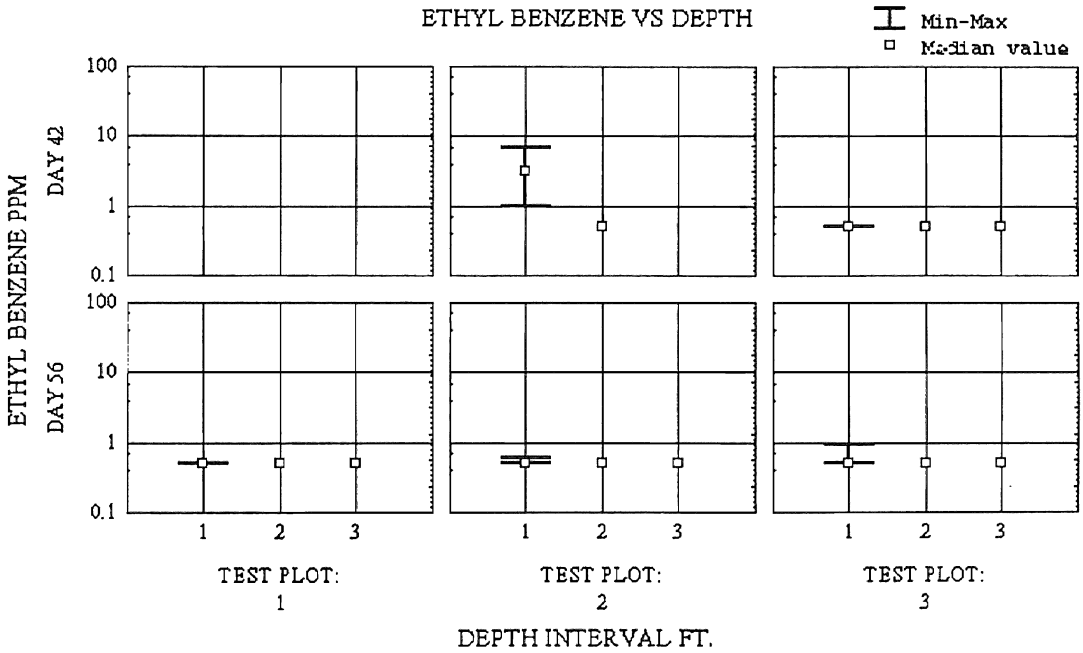


Figure 45. Ethyl Benzene vs. Depth, Days 42 and 56

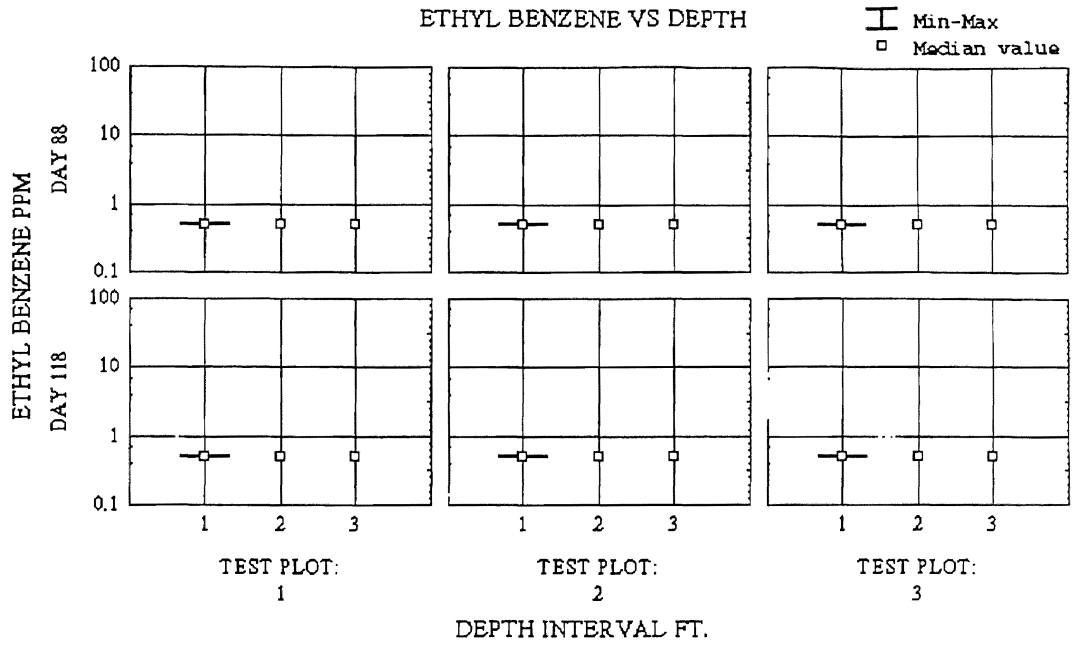


Figure 46. Ethyl Benzene vs. Depth, Days 88 and 118

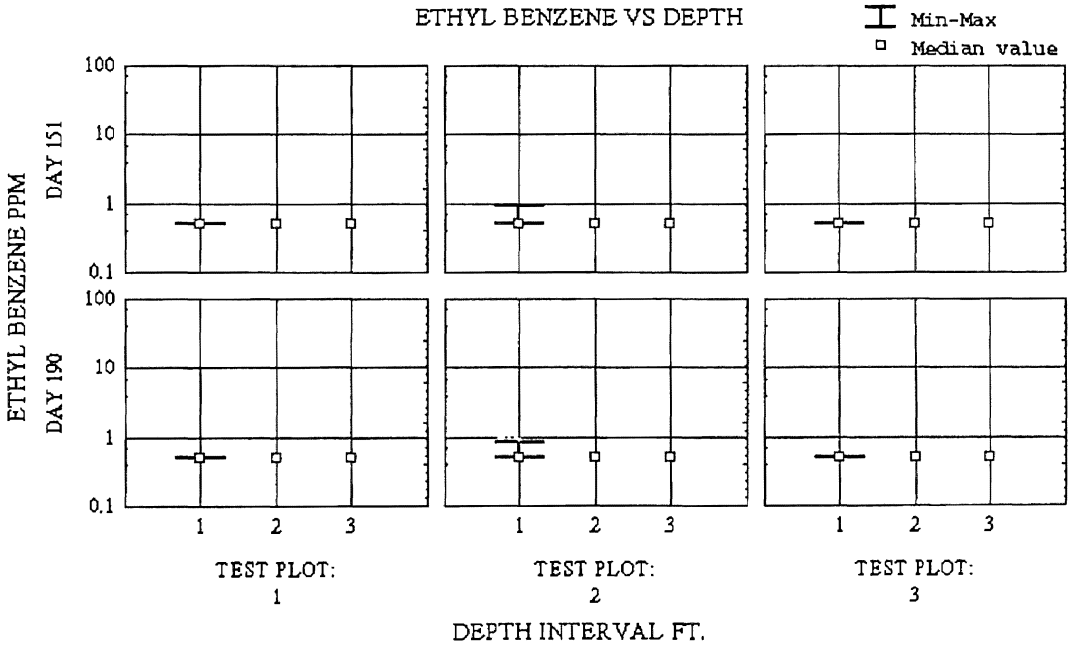


Figure 47. Ethyl Benzene vs. Depth, Days 150 and 190

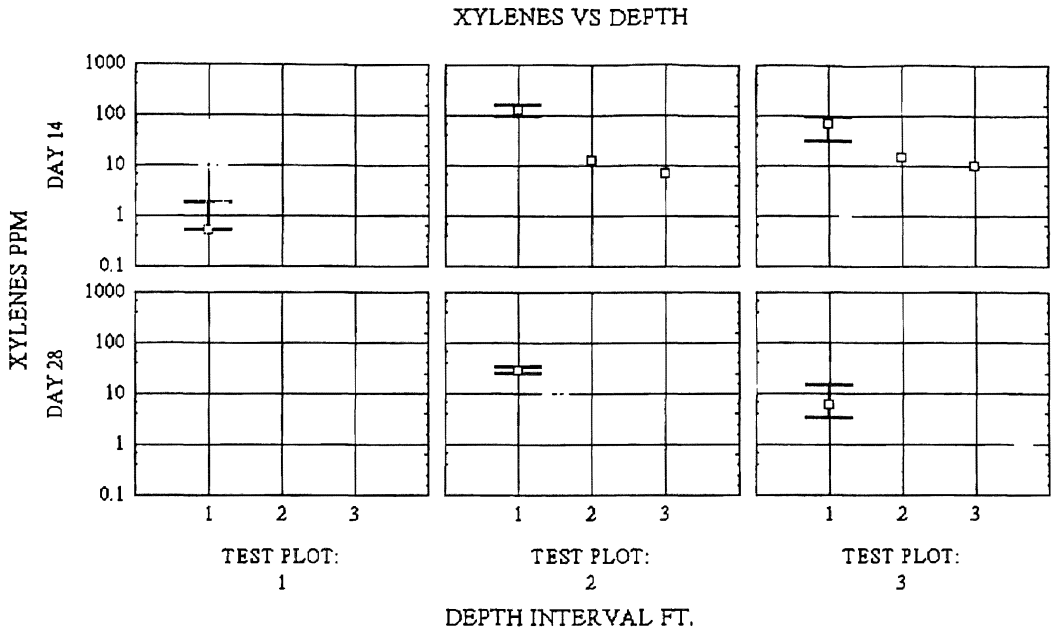


Figure 48. Xylenes vs. Depth, Days 14 and 28

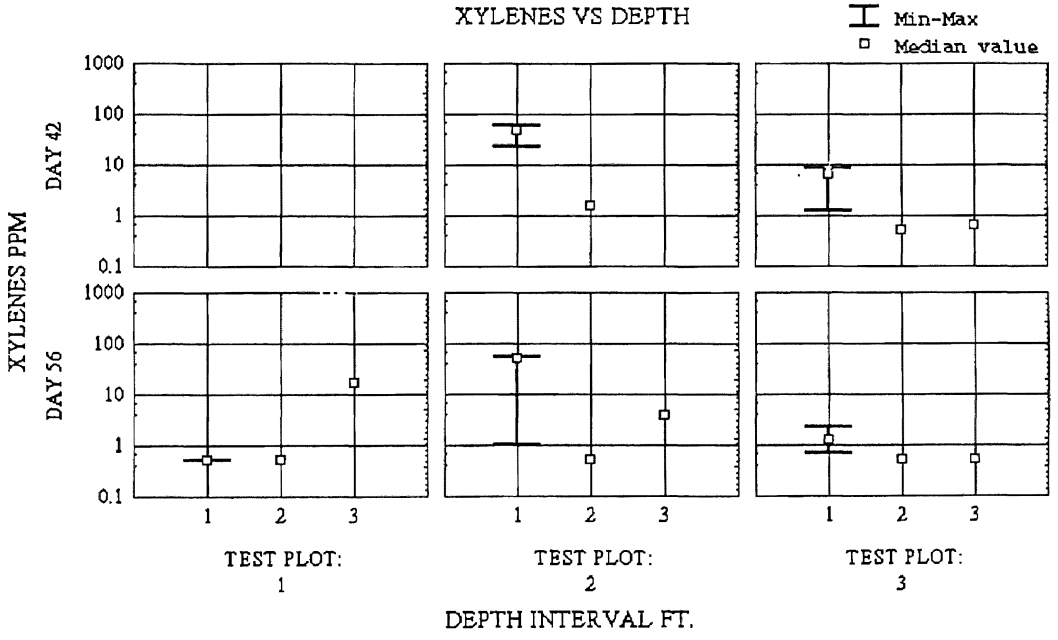


Figure 49. Xylenes vs. Depth, Days 42 and 56

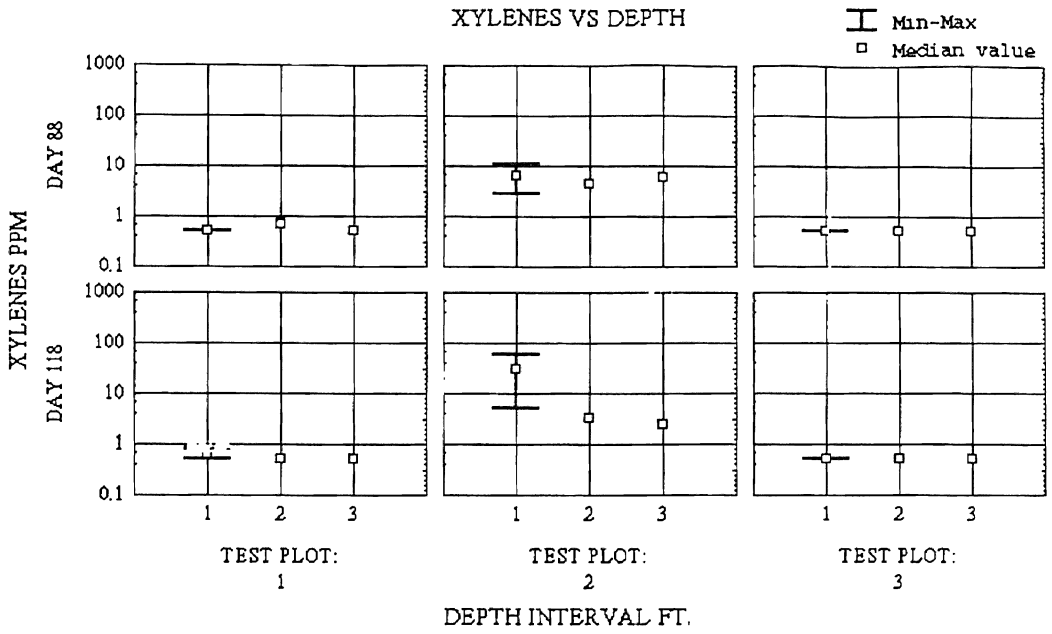


Figure 50. Xylenes vs. Depth, Days 88 and 118

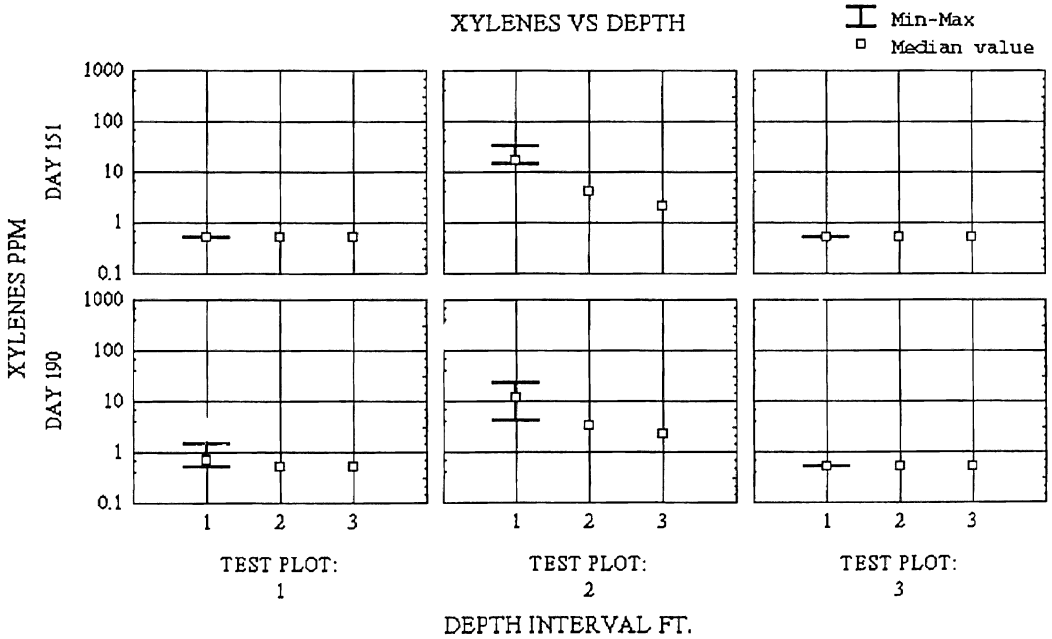


Figure 51. Xylenes vs. Depth, Days 151 and 190

APPENDIX D

MICROTOX BASIC PROTOCOL EFFECTIVE CONCENTRATIONS

TABLE XI

MICROTOX®FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
0-1-1-1	130.24	8.5E+01	2.0E+02	28.31	2.4E+01	3.4E+01	11.59	9.9E+00	1.4E+01	0.83	0.46	1.48	0.9047
0-1-2-1	255.78	9.2E+00	7.1E+03	57.16	1.0E+01	3.1E+-2	23.78	9.1E+00	6.2E+01	1.78	0.12	25.71	0.8081
0-1-3-1	242.78	6.1E+01	9.7E+02	30.25	1.9E+01	4.9E+01	8.95	5.6E+00	1.4E+01	0.24	0.03	1.95	0.6498
0-1-4-1	56.89	2.0E+01	1.6E+02	18.25	1.1E+01	3.2E+01	9.39	4.9E+00	1.8E+01	1.31	0.20	8.44	1.156
0-2-1-1	29.97	1.3E+01	7.1E+00	16.67	8.6E+00	3.2E+01	11.83	5.8E+00	2.4E+01	4.29	1.13	16.29	2.1802
0-2-2-1	7.09	6.9E+00	7.3E+00	0.95	8.8E-01	1.0E+00	0.30	2.6E-01	3.3E-01	0.01	0.01	0.01	0.6912
0-2-3-1	15.78	8.4E+00	3.0E+01	1.19	1.2E-01	1.2E+01	0.26	7.8E-03	8.9E+00	0.00	0.00	4.32	0.4981
0-2-4-1	39.12	2.5E+01	6.1E+01	18.34	1.4E+01	2.5E+01	11.78	8.6E+00	1.6E+01	3.18	1.62	6.24	1.8028
2-2-C-2	225.93	3.9E+01	1.3E+03	17.26	1.1E+01	2.8E+01	3.83	1.4E+00	1.1E+01	0.04	0.00	1.93	0.5165
2-2-C-3	83.04	4.2E+01	1.6E+02	5.18	3.1E+00	8.6E+00	1.02	3.5E-01	3.0E+00	0.01	0.00	0.15	0.4923
0-3-1-1	35.15	3.3E+01	3.8E+01	8.37	7.8E+00	9.0E+00	3.62	3.2E+00	4.1E+00	0.30	0.23	0.40	0.966
0-3-2-1	20.00	1.9E+01	2.1E+01	5.91	5.4E+00	6.5E+00	2.55	2.5E+00	3.3E+00	0.27	0.27	0.46	1.1374
0-3-3-1	39.03	1.4E+01	1.1E+02	17.888.	8.1E+00	3.9E+01	11.33	3.6E+00	3.6E+00	2.94	0.19	45.33	1.7581
0-3-4-1	27.55	2.4E+01	3.2E+01	6.39	5.3E+00	7.6E+00	2.72	2.0E+00	3.6E+00	0.22	0.11	0.42	0.9464
2-3-C-2	45.73	1.6E+01	1.3E+02	17.45	9.4E+00	3.2E+01	9.93	4.8E+00	2.0E+01	1.88	0.30	11.85	1.3429
2-3-C-3													
3-2-1-1	1568	8.4E+00	2.9E+01	1.10	1.1E-01	1.1E+01	0.23	6.5E-03	8.2E+00	0.00	0.00	3.65	0.4852
3-2-2-1	45.03	3.2E+01	6.3E+01	0.39	1.5E-01	1.0E+00	0.02	4.6E-03	1.3E-01	0.00	0.00	0.00	0.2899

TABLE XI

MICROTOX®FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
3-2-3-1	10.12	8.5E+00	1.2E+01	0.40	1.9E-01	8.3E-01	0.06	2.0E-02	1.8E-01	0.00	0.00	0.00	0.427
3-2-4-1	39.42	2.0E+01	7.8E+01	4.21	1.7E+00	1.0E+01	1.14	2.3E-01	5.6E+00	0.02	0.00	1.06	0.5978
3-3-1-1	205.96	6.0E+01	1.1E+02	55.20	2.8E+01	1.1E+02	25.55	1.7E+01	3.9E+01	2.62	1.06	6.49	1.0291
3-3-2-1	616.93	3.6E+01	1.0E+04	53.92	1.8E+01	1.6E+02	12.68	7.0E+00	2.3E+01	0.18	0.01	5.64	0.5327
3-3-3-1	184.04	4.3E+01	7.8E+02	48.94	2.3E+01	1.1E+02	22.55	1.4E+01	3.6E+01	2.28	0.70	7.41	1.0117
3-3-4-1	577.94	4.8E+01	7.0E+03	56.99	2.1E+01	1.6E+02	14.70	8.8E+00	2.5E+01	0.27	0.02	4.52	0.5699
4-2-1-1	33.84	2.8E+01	4.1E+01	2.95	2.1E+00	4.2E+00	0.71	3.9E-01	1.3E+00	0.01	0.00	0.04	0.566
4-2-2-1	160.23	3.9E+01	6.6E+02	13.88	8.9E+00	2.2E+01	3.32	1.2E+00	9.1E+00	0.05	0.00	1.43	0.5469
4-2-3-1	9.46	7.3E+00	1.2E+01	1.28	6.1E-01	2.7E+00	0.40	1.4E-01	1.1E+00	0.01	0.00	0.09	0.6887
4-2-4-1	46.08	3.0E+01	7.0E+01	8.52	6.2E+00	1.2E+01	3.17	1.8E+00	5.6E+00	0.17	0.04	0.74	0.8123
4-2-C-2	86.96	4.1E+01	1.8E+02	5.90	3.6E+00	9.8E+00	1.22	4.2E-01	3.6E+00	0.01	0.01	0.21	0.5066
4-3-1-1	67.41	2.7E+01	1.7E+02	18.16	1.2E+01	2.8E+01	8.43	4.9E+00	1.5E+01	0.87	0.16	4.65	1.0227
4-3-2-1	52.25	1.3E+01	2.1E+02	27.97	1.1E+01	7.1E+01	19.40	9.1E+00	4.2E+01	6.58	2.09	20.73	2.0028
4-3-3-1	45.95	1.2E+00	1.8E+03	27.35	2.6E+00	2.9E+02	20.19	2.1E+00	1.9E+02	8.23	0.08	890.63	2.4189
4-3-4-1	38.13	1.4E+01	1.0E+02	14.33	7.6E+00	2.7E+01	8.09	3.4E+00	1.9E+01	1.49	0.18	12.30	1.3142
4-3-C-2	63.13	4.7E+01	8.5E+01	11.99	1.0E+01	1.4E+01	4.54	3.4E+00	6.0E+00	0.26	0.12	0.56	0.8315
4-3-C-3	226.65	3.5E+01	1.5E+03	34.72	1.7E+01	7.2E+01	11.58	6.6E+00	2.0E+01	0.45	0.04	5.11	0.7045
5-1-1-1	113.76	4.6E+01	2.8E+02	34.63	2.2E+01	5.5E+01	17.27	1.2E+01	2.4E+01	2.21	0.89	5.48	1.1428
5-1-2-1	214.30	1.7E+02	2.7E+02	28.10	2.6E+01	3.1E+01	8.56	7.8E+00	9.4E+00	0.25	0.17	0.37	0.6818

TABLE XI

MICROTOX® FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
5-1-3-1	845.53	3.4E+01	2.1E+04	48.99	1.7E+01	1.4E+02	9.26	4.5E+00	1.9E+01	0.07	0.00	5.29	0.4559
5-1-4-1	214.48	5.4E+01	8.4E+02	28.79	1.8E+01	4.7E+01	8.89	5.5E+00	1.4E+01	0.28	0.03	2.22	0.6718
5-1-C-2	510.40	5.3E+01	4.9E+03	70.74	2.4E+01	2.1E+06	22.26	1.3E+01	3.8E+01	0.73	0.10	5.55	0.5762
5-1-C-3	358.20	5.5E+01	2.4E+03	42.33	2.0E+01	8.8E+01	12.14	7.6E+00	2.0E+01	0.30	0.03	3.23	0.6258
5-2-1-1	75.83	2.8E+01	2.1E+02	8.78	5.0E+00	1.5E+01	2.49	7.8E-01	7.9E+00	0.06	0.00	1.54	0.6207
5-2-2-1	40.79	2.8E+01	5.8E+01	7.65	5.6E+00	1.0E+01	2.87	1.7E+00	5.0E+00	0.16	0.04	0.63	0.8204
5-2-3-1	43.85	3.3E+01	5.8E+01	6.64	5.2E+00	8.5E+00	2.20	14E+00	3.5E+00	0.08	0.03	0.26	0.731
5-2-4-1	14.10	9.2E+00	2.2E+01	2.08	6.2E-01	7.0E+00	0.68	1.1E-01	4.2E+00	0.02	0.00	0.95	0.7009
5-2-C-2	485.37	1.2E+02	2.0E+03	45.67	2.7E+01	7.8E+01	11.46	8.1E+00	1.6E+01	0.19	0.03	1.20	0.5762
5-2-C-3	316.42	1.6E+02	4.5E+01	35.42	2.8E+01	4.5E+01	9.84	8.0E+00	1.2E+01	0.22	0.08	0.59	0.6297
5-3-1-1	192.55	1.9E+01	1.9E+03	42.15	1.4E+01	1.3E+02	17.33	8.9E+00	3.4E+01	1.25	0.12	13.17	0.8408
5-3-2-1	192.68	1.8E+01	2.0E+03	41.14	1.4E+01	1.2E+02	16.67	8.5E+00	3.3E+01	1.15	0.10	13.60	0.8249
5-3-3-1	129.50	5.6E+01	3.0E+02	42.31	2.7E+01	6.7E+01	21.99	1.6E+01	3.0E+01	3.18	1.63	6.20	1.2207
5-3-4-1	101.59	2.2E+01	4.6E+02	41.21	1.6E+01	1.0E+02	24.31	1.3E+01	4.7E+01	5.10	1.81	14.37	1.4461
5-3-C-2	353.32	2.5E+01	4.9E+03	95.24	1.9E+01	4.7E+02	44.23	1.6E+01	1.3E+02	4.58	1.38	15.17	0.9851
5-3-C-3	434.38	1.0E+02	1.8E+03	41.25	2.5E+01	6.9E+01	10.41	7.2E+00	1.5E+01	0.18	0.03	1.21	0.5778
6-1-1-1	1.9E+04	6.7E+01	5.5E+06	603.15	3.2E+01	1.1E+04	79.54	1.9E+01	3.3E+02	0.20	0.01	6.77	0.3748
6-1-2-1	1762.74	4.1E+02	7.6E+03	246.75	1.0E+02	5.9E+02	78.12	4.5E+01	1.3E+02	2.60	1.42	4.77	0.6979
6-1-3-1	2.1E+04	6.1E+02	6.9E+05	746.27	1.1E+02	5.1E+03	107.31	3.9E+01	2.9E+02	0.35	0.05	2.32	0.4077

TABLE XI

MICROTOX®FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
6-1-4-1	2289.12	5.0E+02	1.0E+04	310.32	1.2E+02	7.9E+02	96.41	5.3E+01	1.7E+02	3.04	1.75	5.28	0.6869
6-1-C-2	188.23	3.8E+01	9.2E+02	43.97	2.0E+01	9.6E+01	18.78	1.2E+01	3.0E+01	1.52	0.33	6.92	0.916
6-1-C-3	269.32	1.5E+02	4.7E+02	72.36	5.2E+01	1.0E+02	33.55	2.7E+01	4.1E+01	3.45	2.50	4.78	1.0508
6-2-1-1	68.75	4.4E+01	1.1E+02	6.31	4.6E+00	8.7E+00	1.56	8.0E-01	3.0E+00	0.03	0.00	0.15	0.5858
6-2-2-1	32.39	2.3E+01	4.6E+01	2.72	1.4E+00	5.2E+00	0.64	2.1E-01	2.0E+00	0.01	0.00	0.11	0.5527
6-2-3-1	19.88	1.6E+01	2.5E+01	6.01	4.3E+00	8.4E+00	2.99	4.9E+00	4.9E+00	0.38	0.13	1.06	1.15
6-2-4-1	25.34	2.2E+01	2.9E+01	2.51	1.9E+00	2.9E+01	0.65	4.0E-01	1.0E+00	0.01	0.00	0.03	0.5982
6-2-C-2	33.68	2.8E+01	4.1E+01	15.20	1.3E+01	1.8E+01	9.54	8.0E+00	1.1E+01	2.41	1.65	3.53	1.736
6-2-C-3	14.37	1.2E+01	1.7E+01	1.61	9.3E-01	2.8E+00	0.45	2.0E-01	1.0E+00	0.01	0.00	0.05	0.6299
6-4-3-2	100.66	4.6E+01	2.2E+02	42.07	2.6E+01	6.8E+01	25.25	1.8E+01	3.6E+01	5.58	3.37	9.26	1.5632
6-3-3-1	115.40	4.3E+01	3.1E+02	43.83	2.4E+01	7.9E+01	24.88	1.7E+01	3.7E+01	4.66	2.39	9.08	1.3998
6-3-4-1	133.88	1.6E+01	1.1E+03	50.34	1.4E+01	1.8E+02	28.41	1.2E+01	6.8E+01	5.23	1.51	18.15	1.296
6-3-C-2	45.79	2.2E+01	9.6E+01	22.21	1.4E+01	3.6E+01	14.54	9.4E+00	2.3E+01	4.16	1.72	10.03	1.8504
6-3-C-3	118.42	1.4E+01	1.0E+03	41.54	1.2E+01	1.4E+02	22.51	9.9E+00	5.1E+01	3.68	0.71	19.09	1.1933
7-1-1-1	1.6E+04	9.6E-01	2.7E+08	605.86	3.4E+00	1.1E+05	89.13	6.6E+00	1.2E+03	0.31	0.01	83.42	0.3498
7-1-2-1	733.15	2.3E+02	2.4E+03	131.37	6.6E+01	2.6E+02	48.05	3.2E+01	7.2E+01	2.46	1.33	4.54	0.7983
7-1-3-1													
7-1-4-1	785.10	2.5E+01	2.4E+04	1.3E+06	1.9E+01	8.5E+02	43.64	1.5E+01	1.3E+02	1.86	0.26	13.23	0.7037
7-1-C-2	1.1E+04	2.7E+02	4.1E+05	751.18	8.3E+01	6.8E+03	160.49	4.1E+01	6.3E+02	1.67	0.44	6.34	0.5079

TABLE XI

MICROTOX®FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
7-1-C-3	1109.99	1.9E+01	6.6E+04	182.05	1.6E+01	2.0E+03	63.23	1.4E+01	2.8E+02	2.77	0.46	16.74	0.699
7-2-1-1	14.04	9.0E+00	2.2E+01	2.76	9.2E-01	8.3E+00	1.06	2.1E-01	5.3E+00	0.06	0.00	1.56	0.8225
7-2-2-1	7.16	4.7E+00	1.1E+01	0.93	3.1E-01	2.8E+00	0.28	6.1E-02	1.3E+00	0.01	0.00	0.15	0.67
7-2-3-1	5.80	4.9E+00	6.9E+00	0.69	4.5E-01	1.1E+00	0.20	1.1E-01	3.5E-01	0.00	0.00	0.01	0.6491
7-2-4-1	2.74	1.7E+00	4.3E+00	0.15	5.0E-02	4.7E-01	0.03	6.2E-03	1.3E-01	0.00	0.00	0.00	0.4785
7-2-C-2	61.85	3.7E+01	1.0E+02	7.43	5.2E+00	1.1E+01	2.15	1.1E+00	4.3E+00	0.06	0.01	0.36	0.6468
7-2-C-3	33.57	1.8E+01	6.4E+01	3.73	1.4E+00	1.0E+01	1.03	1.9E-01	5.7E+00	0.02	0.00	1.23	0.6072
7-3-1-1	169.04	3.2E+01	8.8E+02	52.06	2.0E+01	1.3E+02	26.14	1.4E+01	4.8E+01	3.41	1.08	10.71	1.1231
7-3-2-1	328.87	1.0E+02	1.0E+03	76.99	4.0E+01	1.5E+02	32.93	2.2E+01	4.9E+01	2.67	1.30	5.49	0.9408
7-3-3-1	118.42	9.3E+01	1.5E+02	38.56	3.4E+01	4.4E+01	20.00	1.8E+01	2.2E+01	2.87	2.33	3.54	1.2339
7-3-4-1	109.81	4.4E+01	2.7E+02	35.66	2.2E+01	5.8E+01	18.47	1.3E+01	2.6E+01	2.64	1.12	6.20	1.2077
7-3-C-2	239.74	5.9E+01	9.7E+02	49.58	2.5E+01	9.8E+01	19.72	1.3E+01	2.9E+05	1.29	0.35	4.76	0.8568
7-3-C-3	498.03	7.7E-01	3.2E+05	40.25	4.9E+00	3.3E+02	9.24	1.9E+00	4.5E+01	0.12	0.00	0.24	0.4027
8-1-1-1	8786.47	2.7E+03	2.6E+04	506.77	2.7E+02	9.6E+02	97.47	6.8E+01	1.4E+02	0.74	0.42	1.31	0.4902
8-1-2-1	4315.46	1.0E+00	1.9E+07	378.24	3.1E+00	4.6E+04	91.06	5.6E+00	1.5E+03	1.35	0.03	60.18	0.4607
8-1-3-1	4082.35	3.7E+01	4.6E+05	380.60	2.4E+01	5.9E+03	95.00	1.9E+01	4.9E+02	1.57	0.20	12.19	0.5427
8-1-4-1	1707.30	6.2E+02	4.7E+03	266.26	1.4E+02	5.0E+02	89.79	6.0E+01	1.3E+02	3.61	2.53	5.15	0.7424
8-1-C-2	8996.94	1.4E+03	5.8E+04	543.20	1.9E+02	1.6E+03	105.15	5.8E+01	1.9E+02	0.82	0.33	2.00	0.4893
8-1-C-3	1709.30	1.8E+02	1.6E+04	208.35	5.9E+01	7.4E+02	60.83	2.9E+01	1.3E+02	1.60	0.51	5.01	0.6432

TABLE XI

MICROTOX®FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
8-2-1-1	26.69	1.3E+01	5.3E+01	6.00	2.4E+00	1.5E+01	2.50	5.6E-01	1.1E+01	0.19	0.01	5.58	0.8748
8-2-2-1	37.33	3.3E+01	4.2E+01	8.68	7.8E+00	9.7E+00	3.70	3.1E+00	4.4E+00	0.30	0.19	0.46	0.9492
8-2-3-1	25.61	1.3E+01	5.1E+01	6.03	2.3E+00	1.6E+01	2.59	5.6E-01	1.2E+01	0.21	0.01	6.44	0.8995
8-2-4-1	10.11	6.1E+00	1.7E+01	1.07	2.1E-01	5.4E+00	0.29	2.8E-02	3.0E+00	0.01	0.00	0.57	0.5963
8-2-C-2	355.13	8.0E+01	1.6E+03	32.17	2.0E+01	5.2E+01	7.90	4.9E+00	1.3E+01	0.12	0.01	1.23	0.5640
8-2-C-3	243.54	4.3E+01	1.4E+03	18.79	1.2E+01	3.0E+01	4.20	1.6E+00	1.1E+01	0.05	0.00	1.77	0.5199
8-3-1-1	60.21	2.9E+01	1.3E+02	20.03	1.8E+01	4.4E+01	17.92	1.2E+01	2.6E+01	4.78	2.41	9.46	1.7697
8-3-2-1	19.53	1.4E+01	2.7E+01	7.94	5.2E+00	1.2E+01	4.69	2.6E+00	8.5E+00	0.99	0.30	3.28	1.5121
8-3-3-1	140.72	3.8E+01	5.1E+02	44.87	2.2E+01	9.3E+01	22.99	1.4E+01	3.7E+01	3.18	1.16	8.70	1.1723
8-3-4-1	45.32	1.9E+01	1.1E+02	22.20	1.3E+01	3.9E+01	14.62	8.7E+00	2.5E+01	4.26	1.51	11.99	1.8499
8-3-C-2	159.29	2.6E+00	9.7E+03	56.15	6.5E+00	4.9E+02	30.51	8.4E+00	1.1E+02	5.03	0.20	126.98	1.2969
8-3-C-3	298.87	1.2E+01	7.2E+03	87.39	1.2E+01	6.2E+02	42.56	1.2E+01	1.5E+02	5.07	1.22	21.18	1.0068
9-1-1-1	594.97	5.4E+01	6.6E+03	75.02	2.4E+01	2.3E+02	22.34	1.3E+01	3.8E+01	0.62	0.07	5.39	0.6403
9-1-2-1	7.0E+04	2.7E-03	1.8E+12	4060.13	6.4E-02	2.6E+08	769.84	4.0E-01	1.5E+06	5.64	0.24	132.66	0.4745
9-1-3-1	3145.54	1.0E-04	1.0E+04	481.27	9.6E-03	2.4E+07	160.50	1.3E-01	1.9E+05	6.24	0.05	794.71	0.684
9-1-4-1	2.0E+04	5.8E+03	6.6E+04	1005.78	4.9E+02	2.1E+03	176.79	1.2E+02	2.7E+02	1.03	0.64	1.67	0.465
9-1-C-2	5.2E+04	1.3E+02	2.0E+07	1783.72	5.3E+01	6.0E+04	249.28	3.0E+01	2.0E+03	0.74	0.07	7.59	0.3893
9-1-C-3	1.2E+04	6.3E+02	2.3E+05	800.77	1.4E+02	4.7E+03	164.88	5.6E+01	4.9E+02	1.54	0.52	4.55	0.5026
9-2-1-1	34.73	2.1E+01	5.8E+01	5.99	3.4E+00	1.1E+01	2.14	7.9E-01	5.8E+00	0.10	0.01	1.11	0.7705

TABLE XI

MICROTOX® FIVE MINUTE BASIC PROTOCOL EFFECTIVE CONCENTRATIONS WITH CONFIDENCE INTERVAL AND SLOPE

Sample	EC50 % WSF	95% Conf. Int.		EC20 % WSF	95% Conf. Int.		EC10 % WSF	95% Conf. Int.		EC1 % WS	95% Conf. Int.		Slope
9-2-2-1	21.83	1.7E+01	2.8E+01	2.80	1.5E+00	5.1E+00	0.84	3.2E-01	2.2E+00	0.02	0.00	0.19	0.6673
9-2-3-1	26.85	2.3E+01	3.1E+01	6.19	5.1E+00	7.6E+00	2.63	1.9E+00	3.6E+00	0.21	0.10	0.43	0.9424
9-2-4-1	27.05	2.2E+01	3.3E+01	5.53	4.1E+00	7.4E+00	2.19	1.4E+00	3.5E+00	0.14	0.05	0.40	0.869
9-2-C-2	81.18	3.4E+01	1.9E+02	8.39	5.2E+00	1.4E+01	2.23	8.1E-01	6.1E+00	0.04	0.00	0.76	0.5962
9-2-C-3	82.97	5.8E+01	1.2E+02	17.80	1.5E+01	2.1E+01	7.24	5.8E+00	9.0E+00	0.51	0.25	1.00	0.8971
9-3-1-1	218.84	4.4E+01	1.1E+03	47.19	2.2E+01	1.0E+02	19.24	1.2E+01	3.1E+01	1.35	0.30	6.22	0.8708
9-3-2-1	218.84	3.3E+01	1.0E+03	45.54	1.9E+01	1.1E+02	20.09	1.2E+01	3.4E+01	1.79	0.38	8.48	0.9446
9-3-3-1	98.26	2.8E+-1	3.4E+02	37.99	1.8E+01	7.9E+01	21.79	1.3E+01	3.7E+01	4.21	1.59	11.15	1.3976
9-3-4-1	155.56	1.0E+02	2.4E+02	44.27	3.5E+01	5.6E+01	21.23	1.8E+01	2.5E+01	2.41	1.66	3.51	1.099
9-3-C-2	350.78	2.9E+02	4.2E+02	85.67	7.7E+01	9.5E+01	37.56	3.5E+01	4.0E+01	3.28	2.96	3.64	0.9831
9-3-C-3	852.65	9.1E+01	8.0E+03	126.19	3.7E+01	4.3E+02	41.27	2.1E+01	8.1E+01	1.52	0.39	5.92	0.7025

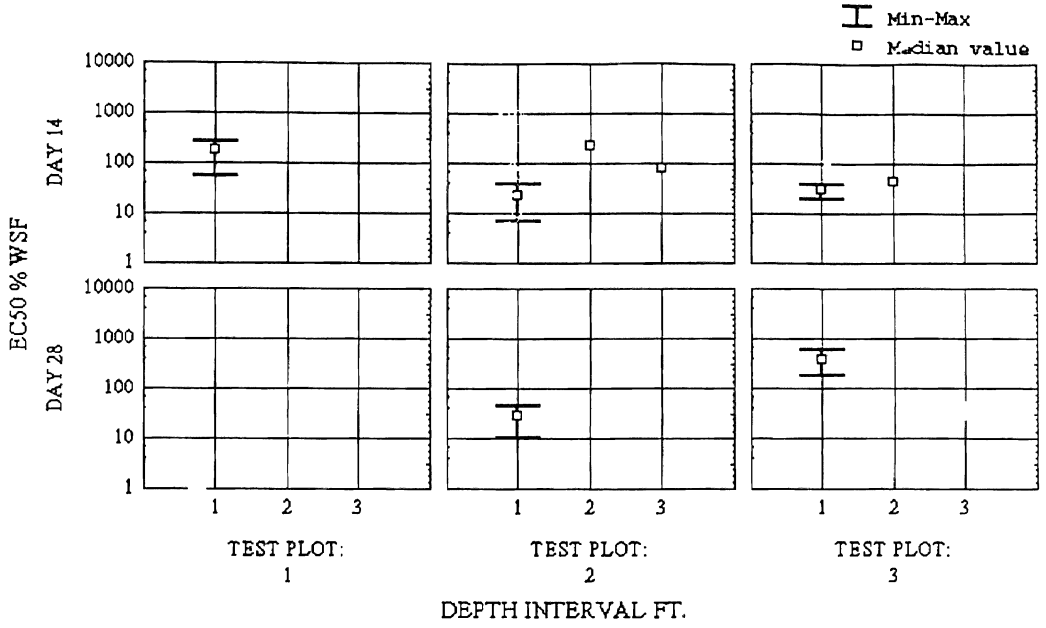


Figure 52. Microtox® EC50 vs. Depth, Days 14 and 28

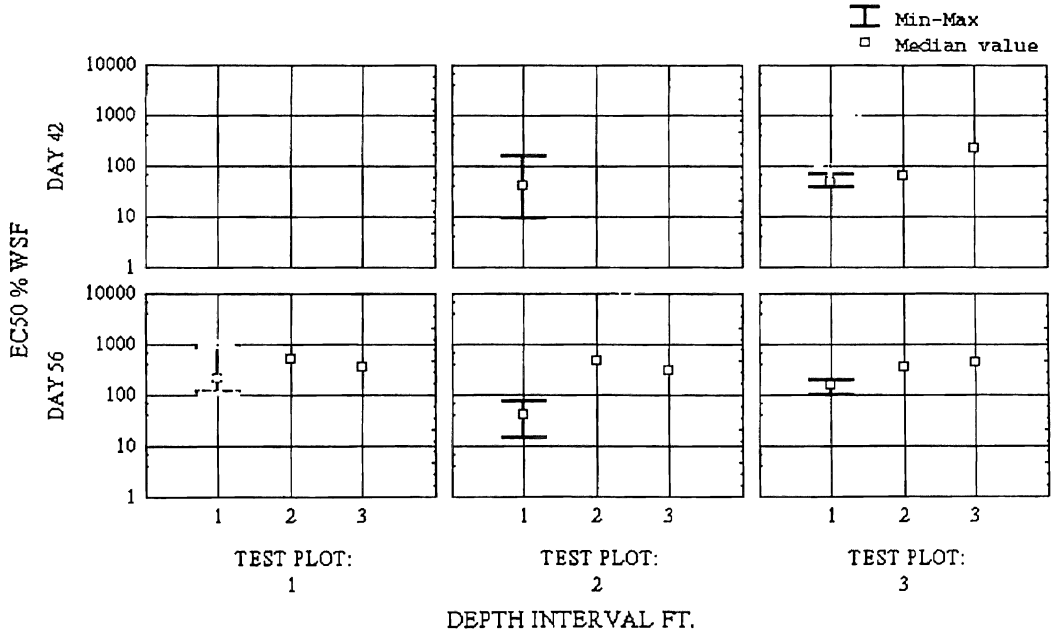


Figure 53. Microtox® EC50 vs. Depth, Days 42 and 56

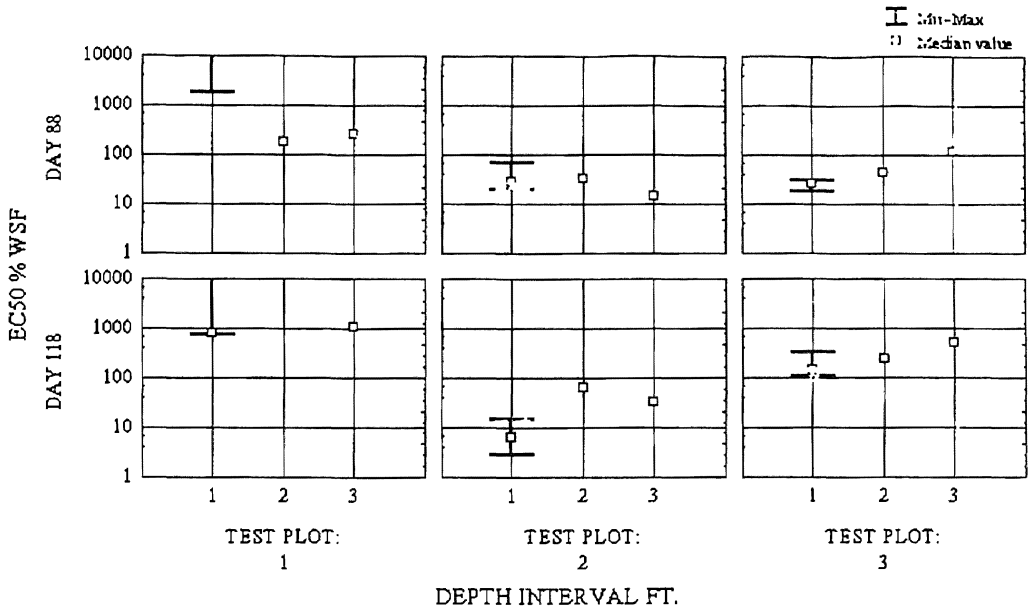


Figure 54. Microtox® EC50 vs. Depth, Days 88 and 118

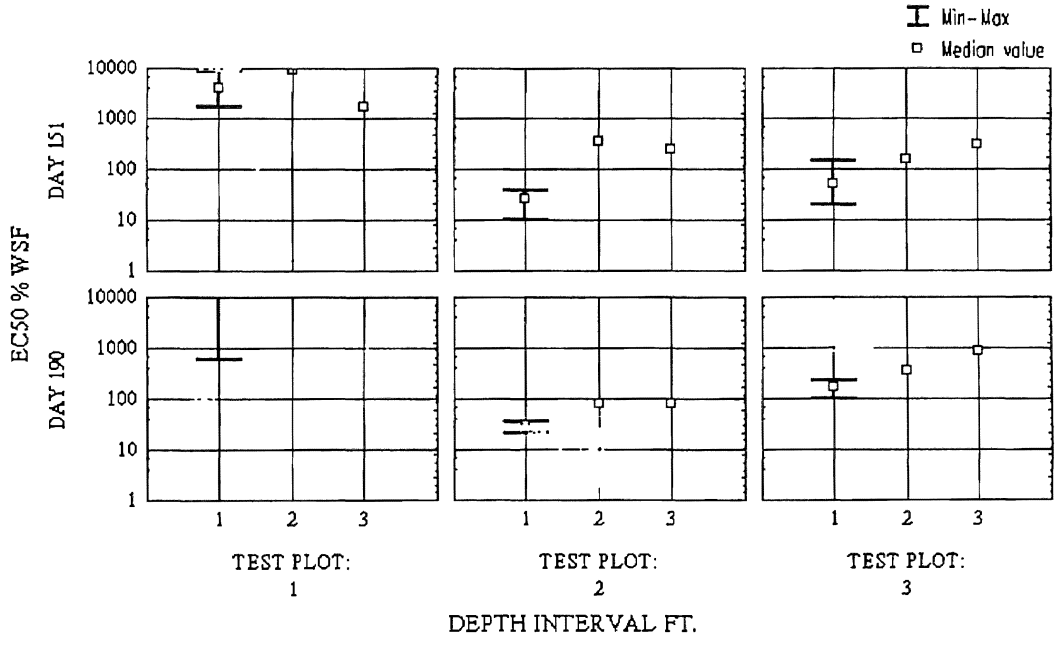


Figure 55. Microtox® EC50 vs. Depth, Days 151 and 190

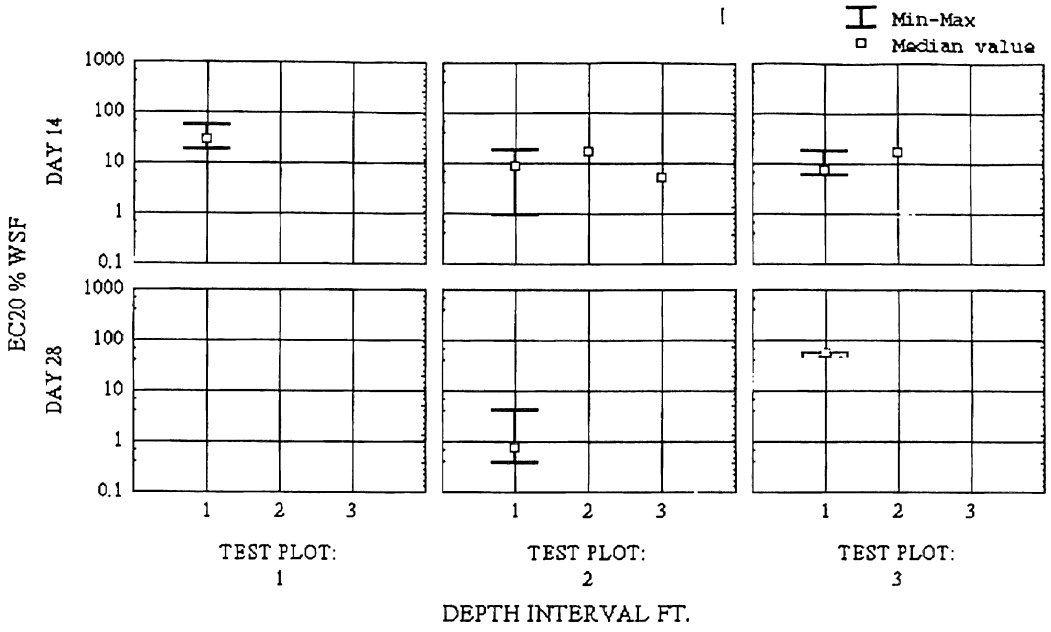


Figure 56. Microtox® EC20 vs. Depth, Days 14 and 28

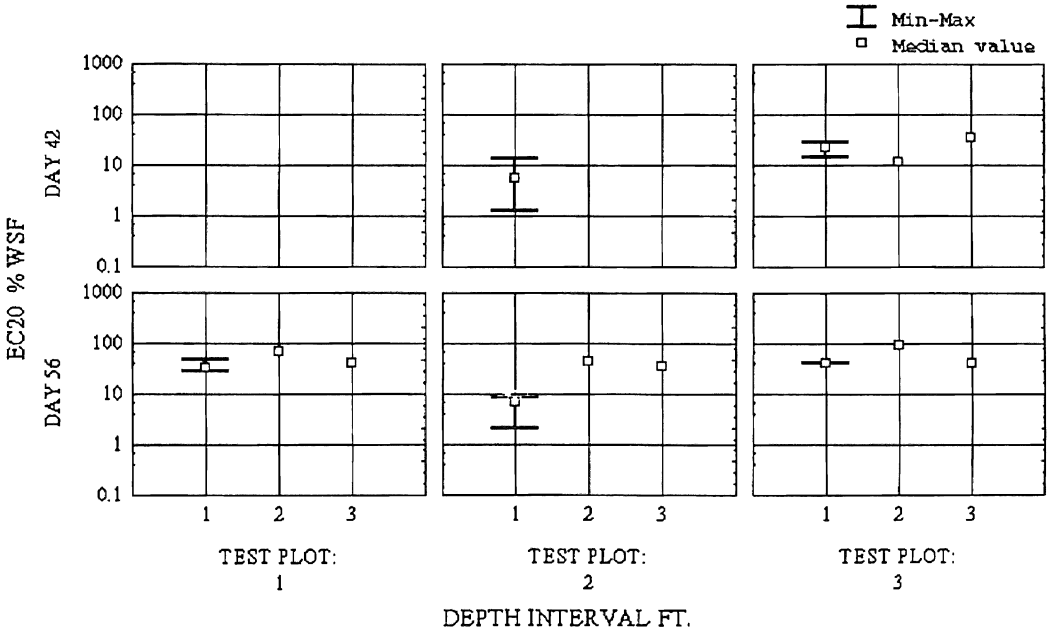


Figure 57. Microtox® EC20 vs. Depth, Days 42 and 56

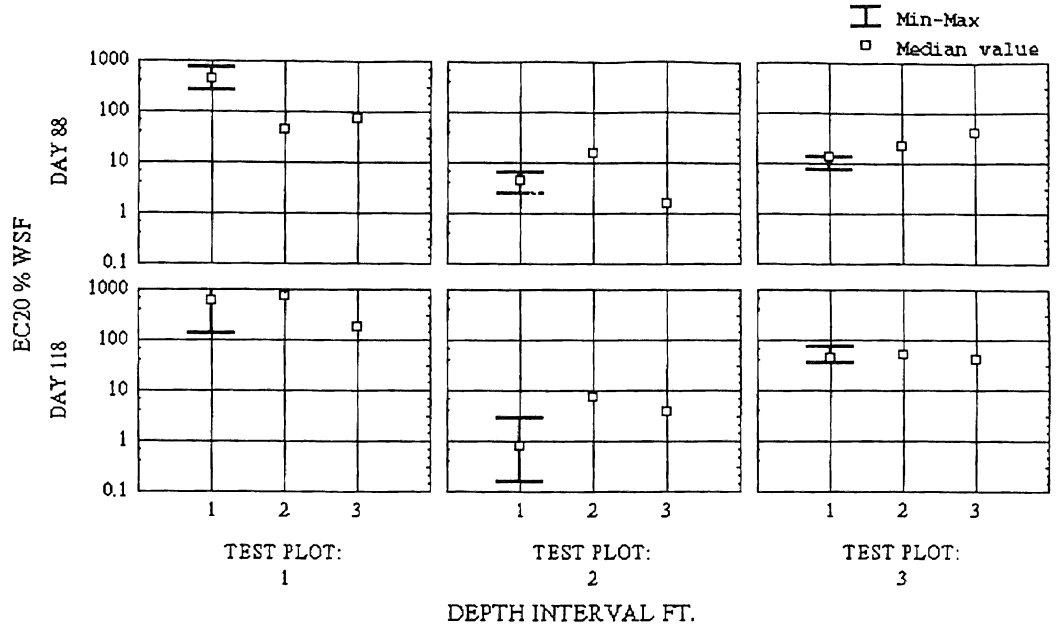


Figure 58. Microtox® EC50 vs. Depth, Days 88 and 118

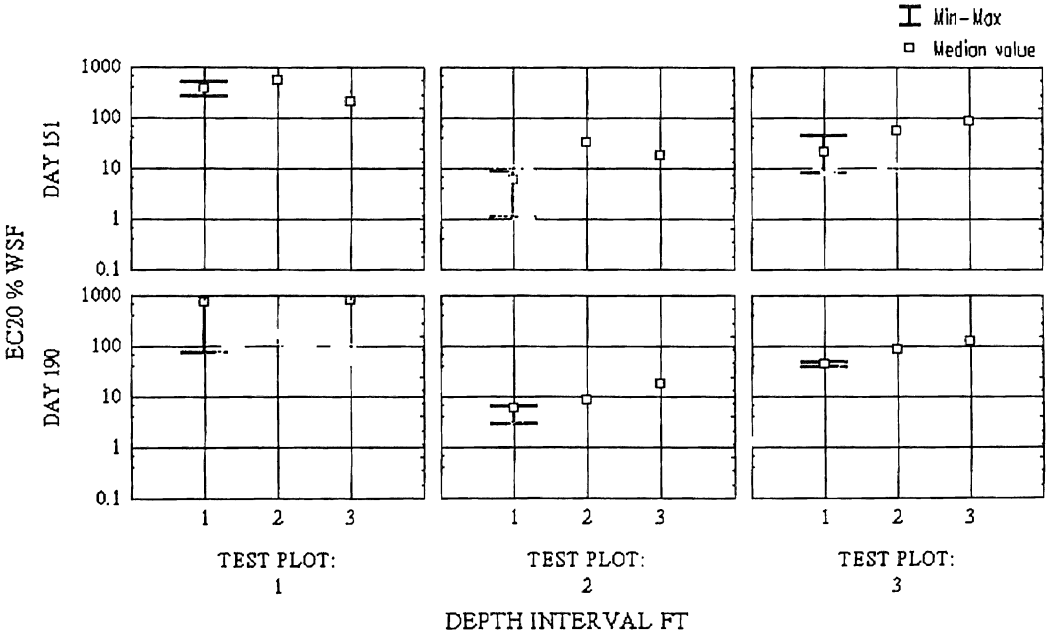


Figure 59. Microtox® EC20 vs. Depth, Days 151 and 190

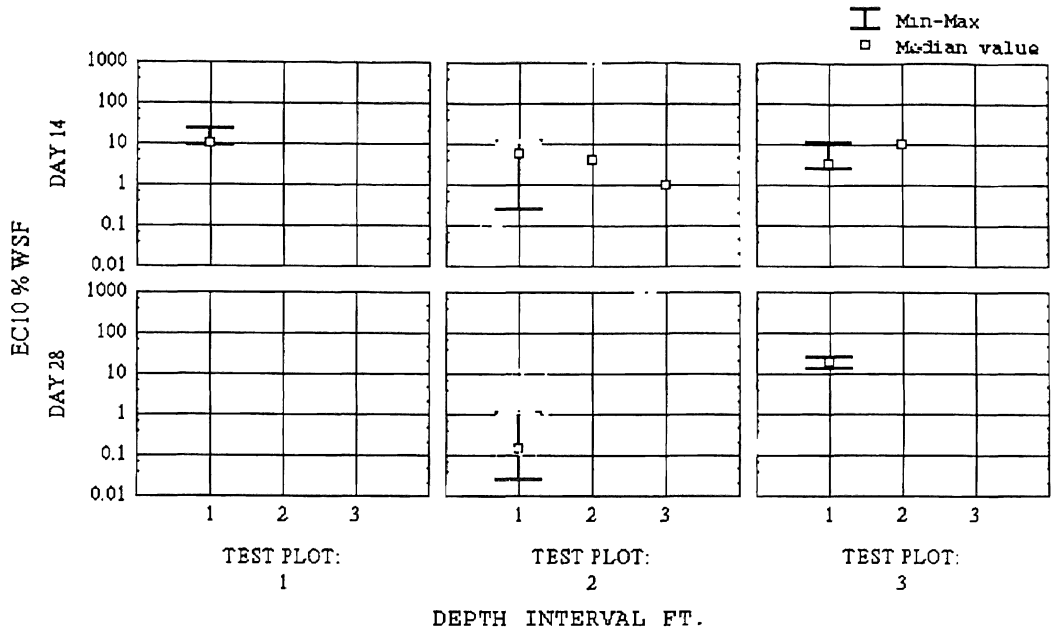


Figure 60. Microtox® EC10 vs. Depth, Days 14 and 28

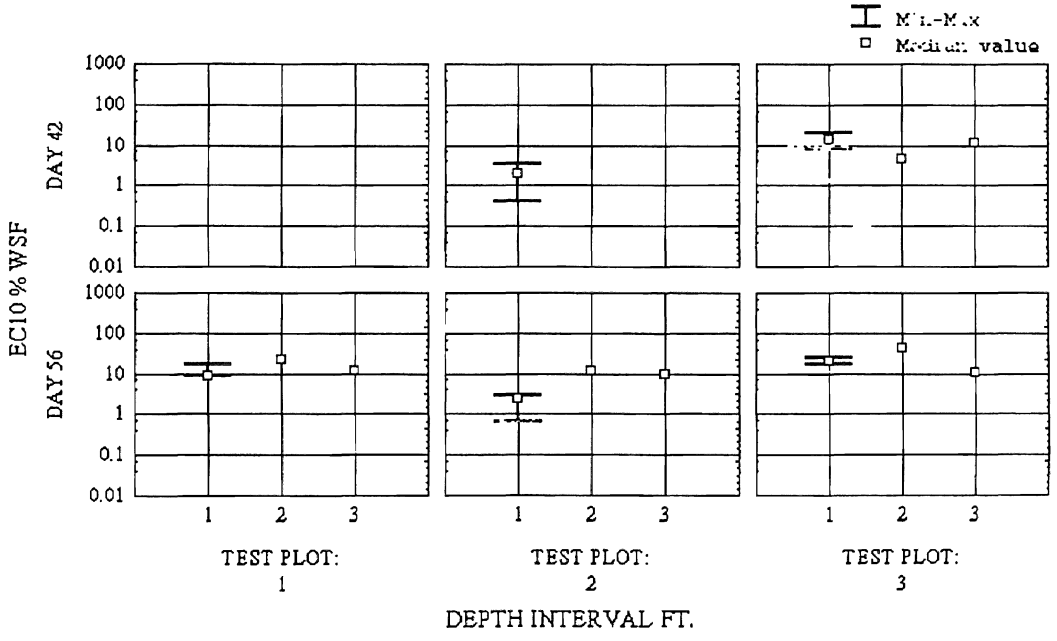


Figure 61. Microtox® EC10 vs. Depth, Days 42 and 56

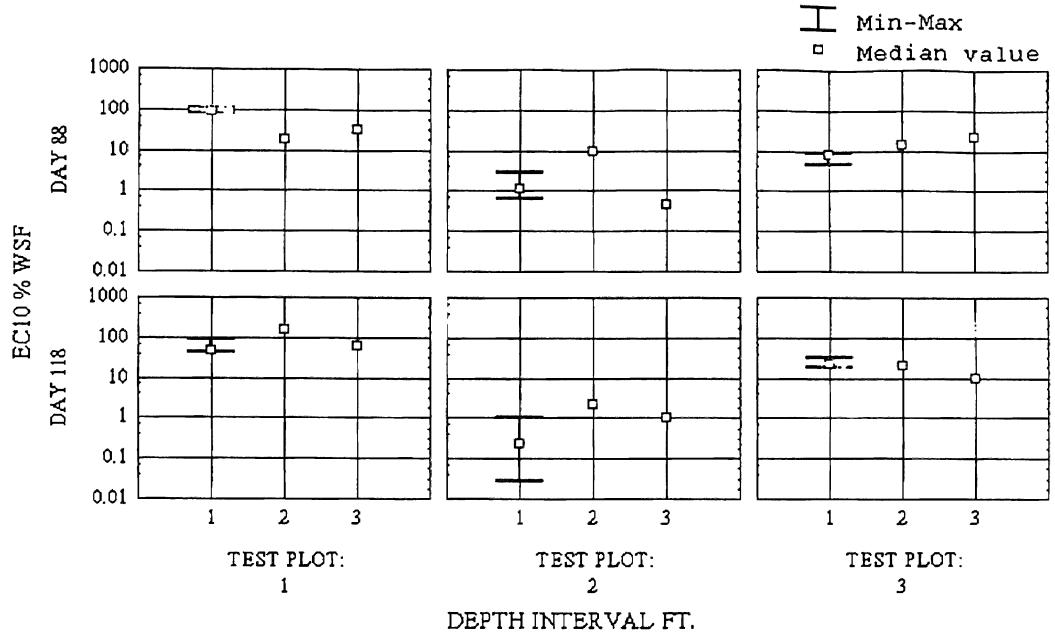


Figure 62. Microtox® EC10 vs. Depth, Days 88 and 118

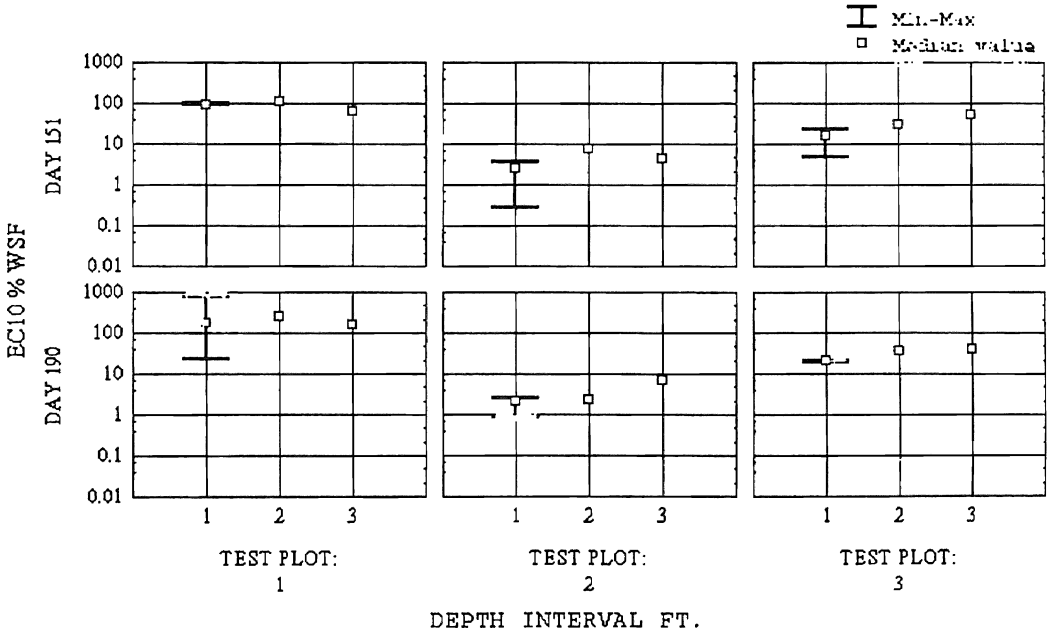


Figure 63. Microtox® EC10 vs. Depth, Days 151 and 190

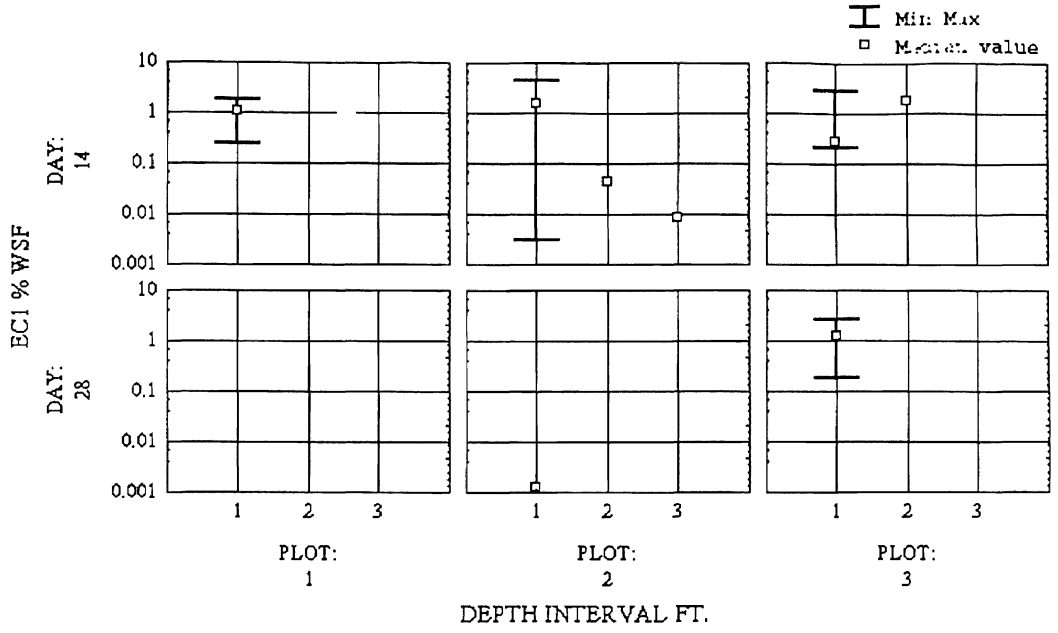


Figure 64. Microtox® EC1 vs. Depth, Days 14 and 28

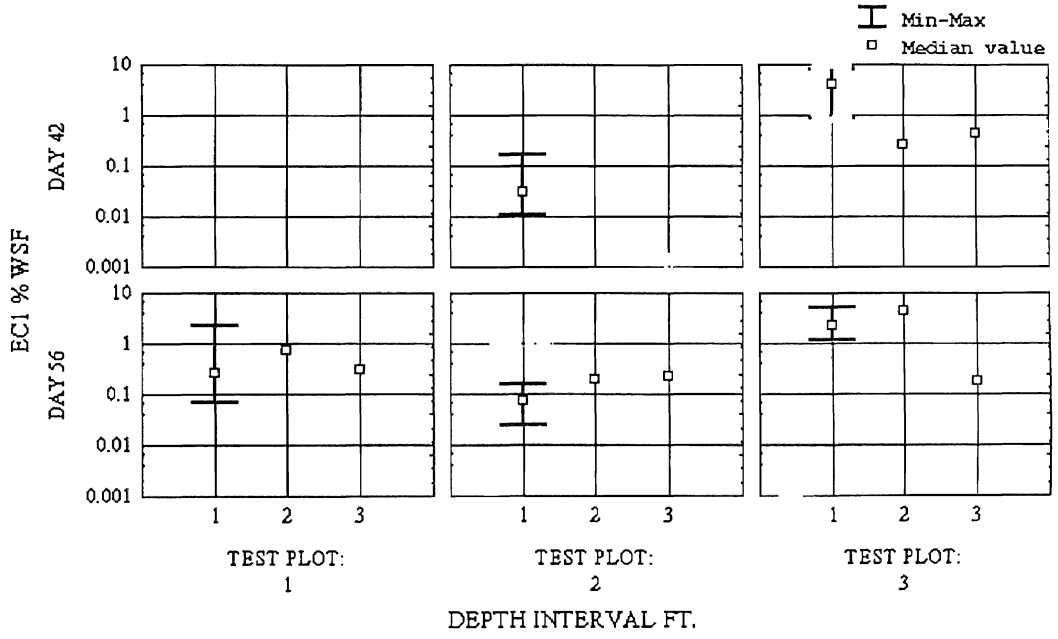


Figure 65. Microtox® EC1 vs. Depth, Days 42 and 56

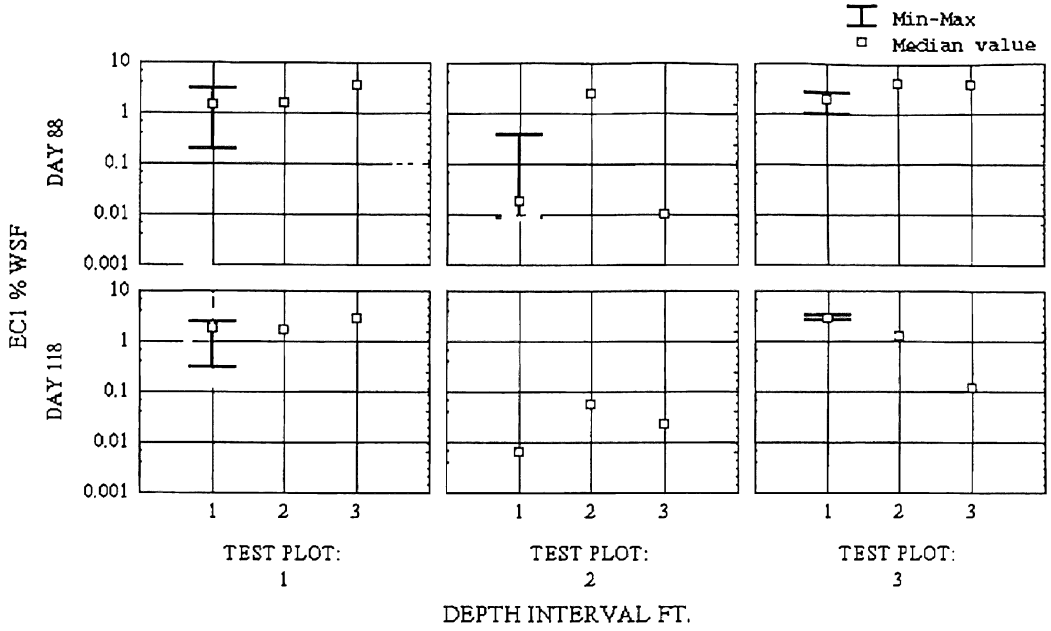


Figure 66. Microtox® EC1 vs. Depth, Days 88 and 118

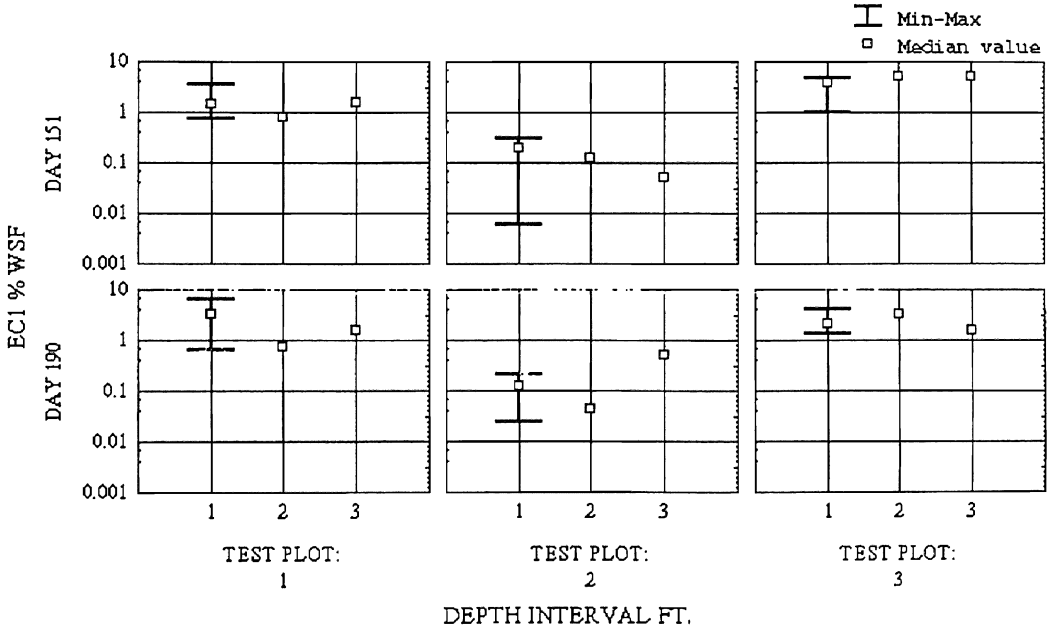


Figure 67. Microtox® EC1 vs. Depth, Days 151 and 190

APPENDIX E

MICROTOX PROTOCOLS USED IN STUDY

MICROTOX® PROTOCOLS USED IN STUDY

The following protocols are all based on Microtox® (Microbics, 1992) protocols and are as performed in this study. It is assumed that the reader has some knowledge of Microtox® analyzer.

Microtox® Basic Protocol

(Using 1 control and 4 1:2 serial dilutions)

Microtox® Analyzer Preparation

8. Place 11 cuvettes in incubator rows A1-A5 & B1-B5 and Reagent well.
9. Add 1,000 ml reconstitution solution to reagent well.
10. Add 500 µl diluent to incubator wells B1 through B5.
11. Add 1,000 µl diluent to incubator wells A1 through A4.

Sample Preparation

1. Add 250 µl MOAS solution to well A5.
2. Add 2,500 µl sample to A5 and mix.
3. Make 1:2 serial dilutions by transferring 1,000 µl from A5 to A4 mix, A4 to A3 mix, A3 to A2 mix.
4. Discard 1,000 µl from well A2 and 750 µl from A5.
5. Wait 5 minutes before testing.

Reagent Preparation

1. Reconstitute a vial of reagent.
2. Mix reagent with 500 µl pipettor 20 times.
3. Add 10 µl to cuvettes B1 through B5.

4. Mix each cuvette, B1 through B5 with 250 µl pipette.
5. Wait 15 minutes after reagent dilution before testing.

All data collection in this study was done by hand and timing by stopwatch. The reader is referred to a Microtox® Protocol Manual for computer preparation and use.

Test Protocol

1. Set timers for 5 and 15 minutes.
2. Place B1 cuvette in READ well. Press set button. Analyzer will self-calibrate for light level.
3. When ready light comes on, make time zero light readings for cuvettes in wells B1 through B5.
4. Immediately make 500 µl transfer from A1 to B1. Start timers and immediately complete 500 µl transfers from A2 to B2, A3 to B3, A4 to B4, A5 to B5.
5. Read and record light readings for cuvettes B1 through B2 at 5 and 15 minutes when prompted by timers.

Data Reduction

1. Data was initially reduced using a Quatro Pro Spreadsheet and its graphing options. Calculations were based on the following basic equations:

   R_t = Correction factor for time t

   I_0 = Light intensity at time 0

   I_t = Light intensity at time t

   R_t = I_t of control (B1)/I_0 of control (B1)

   Calculate Γ values for each dilution as follows:

   $\Gamma = ((R_t \times I_0)/ I_t) - 1$

   Plot log Γ vs. log sample concentrations to determine EC50 concentration at log $\Gamma = 1$.

2. Data was ultimately entered into MTX7 Microbics data reduction soft-ware to calculate EC50, EC20, EC10, EC1, slope and 95% confidence interval.

Note: The above protocol does not depend on the computer to prompt the operator for readings, and precision requires some practice in developing the skill of making transfers and readings at a measured rhythm.

Microtox® LSP Solid -Phase Test Protocol

The following protocol tests in duplicate, using 2 controls and thirteen 1:2 serial dilutions:

Microtox® Analyzer Preparation

1. Place cuvettes in all incubator wells and Reagent well.
2. Add 1,000 µl reconstitution solution to Reagent well.

Test Preparation

1. Place 30 Solid Phase test tubes in holder incubated at 15°C.
   Tubes are arranged in six rows (A-F) containing five tubes each.
2. Pipette 1.5 ml (1,500 µl) of Solid Phase Test Diluent into Solid Phase test tubes.

   A1 through A5
   B1 through B5
   C1 through A5
   D1 through D5
   E1 through E4
   F1 through F4

Sample Preparation

1. Mix sample thoroughly.
2. Weigh 7.0 grams of dry sample.
3. Mix 7.0 g sample and 35 ml of Solid Phase Diluent with a Teflon-covered magnetic stirring bar in a 50 ml serum vial.
4. Mix sample for a minimum of 30 minutes with a magnetic stirrer at a rate which obtains a vortex depth approximately 50% of liquid depth.
5. While stirring, pipette 1.5 ml of sample (from a region adjacent to the wall and about 2 cm above the bottom of the vial) Solid Phase test tubes: E4, E5, F4 and F5.

6. Mix E4 and F4.
7. Make 1:2 serial dilutions by transferring 1.5 ml. (mix after each dilution)

E4 to E3	E3 to E2	E2 to E1	E1 to C5	C5 to C4
C4 to C3	C3 to C2	C2 to C1	C1 to A5	A5 to A4
A4 to A3	Discard 1.5 ml from A3.			
F4 to F3	F3 to F2	F2 to F1	F1 to D5	D5 to D4
D4 to D3	D3 to D2	D2 to D1	D1 to B5	B5 to B4

The resulting concentrations are as follows:

E5	and	F5	19.737%
E4	and	F4	9.868%
E3	and	F3	4.934%
E2	and	F2	2.467%
E1	and	F1	1.234%
C5	and	D5	0.617%
C4	and	D4	0.308%
C3	and	D3	0.154%
C2	and	D2	0.077%
C1	and	D1	0.039%
A5	and	B5	0.019%
A4	and	B4	0.010%
A3	and	B3	0.005%
A2	and	B2	0.000%
A1	and	B1	0.000%

8. Wait 10 minutes for temperature equilibration.

Reagent Preparation

1. Reconstitute a vial of reagent.
2. Mix reagent with 500 µl pipettor 20 times.

All data collection in this study was done by hand and timing by stopwatch. The reader is referred to a Microtox® Protocol Manual for computer preparation and use.

Test Procedure

1. Set timers for 5 and 20 minutes.

2. Start 20-minute timer and immediately transfer 20 µL of reagent to each Solid Phase test tube.
3. Mix each Solid Phase test tube.
4. Insert filter columns into all Solid Phase test tubes.
5. When 20-minute timer signals.
6. Filter each Solid Phase test tube by pressing down filter columns to a point just above settled solids.
7. Start 5-minute timer.
8. Transfer 500µL of filtrate from Solid Phase test tube filter columns to corresponding cuvette in Microtox® analyzer. Filtering and transfers are made in the following order:
   A1, B1, A2, B2, A3, B3, A4, B4, A5, B5, C1, D1, C2, D2, C3, D3, C4, D4, C5, D5, E1, F1, E2, F2, E3, F3, E4, F4, E5, F5.
9. When 5-minute timer sounds, place A1 cuvette in READ well. Press the SET button.
10. 10.Read the I_t (5 minutes) light levels of cuvettes in the following order:
    A1, B1, A2, B2, A3, B3, A4, B4, A5, B5, C1, D1, C2, D2, C3, D3, C4, D4, C5, D5, E1, F1, E2, F2, E3, F3, E4, F4, E5, F5.

Data Reduction

Data recorded in the above procedure was entered by keyboard into the MTX7 data reduction program for the large sample solid phase test.

Note: The above protocol does not depend on the computer to prompt the operator for readings, and precision requires some practice in developing the skill of making transfers and readings at a measured rhythm.

Microtox® NOEC Protocol

Sample pH

1. Measure and record the pH of the sample.
2. In this study, all pH values fell in the interval 6.0-8.0 and was not adjusted before toxicity determination.

Analyzer Preparation

1. Place cuvettes in all wells of incubator and REAGENT WELL.
2. Pipette 750 µL diluent into cuvette wells A2 through A5.
3. Pipette 1250 µL diluent into cuvette well A1.
4. Label five borosilicate test tubes as: B, C, D, E, F.
5. Pipette 2.0 mL diluent into test tube B.
6. Pipette 1.5 mL diluent into test tube C.
7. Pipette 1.0 mL diluent into test tube D.
8. Pipette 0.5 mL diluent into test tube E.

Sample Preparation

1. Add 500.0 mg solid NaCl to test tube F.
2. Add 25.0 ml of sample to test tube F. Mix sample until the NaCl is dissolved.

   NOTE: Some samples require dilution before this step in order to reach the NOEC concentration. This can only be determined by NOEC testing.
3. Add 3.0 mL of osmotically adjusted sample from test tube F to test tube B.
4. Add 3.5 mL of osmotically adjusted sample from test tube F to test tube C.
5. Add 4.0 mL of osmotically adjusted sample from test tube F to test tube D
6. Add 4.5 mL of osmotically adjusted sample from test tube F to test tube B
7. Mix sample B with pipettor, and pipette 750 µL into each cuvette B2 through B5. Pipette 1,250 µL sample B into cuvette in well B1.
8. Mix sample C with pipettor, and pipette 750 µL into each cuvette C2 through C5. Pipette 1,250 µL sample C into cuvette in well C1.
9. Mix sample D with pipettor, and pipette 750 µL into each cuvette D2 through D5. Pipette 1,250 µL sample D into cuvette in well D1.
10. Mix sample E with pipettor, and pipette 750 µL into each cuvette E2 through E5. Pipette 1,250 µL sample E into cuvette in well E1.
11. Mix sample F with pipettor, and pipette 750 µL into each cuvette F2 through F5. Pipette 1,250 µL sample F into cuvette in well F1.

At this point undiluted sample concentrations are: (%)

	1	2	3	4	5
A	00.000	00.000	00.000	00.000	00.000
B	60.000	60.000	60.000	60.000	60.000
C	70.000	70.000	70.000	70.000	70.000
D	80.000	80.000	80.000	80.000	80.000
E	90.000	90.000	90.000	90.000	90.000
F	100.000	100.000	100.000	100.000	100.000

Reagent Preparation

1. Reconstitute a vial of reagent.
2. Mix reagent with 500 µl pipettor 20 times.

All data collection in this study was done by hand and timing by stopwatch. The reader is referred to a Microtox® Protocol Manual for computer preparation and use.

Test Procedure

1. Set and start a timer for 5, 15 and 30 minutes. Then immediately...
2. ...pipette 50 µL of reconstituted reagent into cuvettes A1, B1, C1, D1, E1, F1. Then immediately...
3. ...mix A1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette A1 into each cuvette A2 through A5. Then immediately...
4. ...mix B1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette B1 into each cuvette B2 through B5. Then immediately...
5. ...mix C1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette C1 into each cuvette C2 through C5. Then immediately...
6. ...mix D1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette D1 into each cuvette D2 through D5. Then immediately....
7. ..mix E1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette E1 into each cuvette E2 through E5. Then immediately...
8. ...mix F1 with the 250 µL pipettor 2-3 times, then pipette 250 µL from cuvette F1 into each cuvette F2 through F5. Then immediately...

9. ... mix the following cuvettes by shaking 2-3 times:

A2	through	A5
B2	through	B5
C2	through	C5
D2	through	D5
E2	through	E5
F2	through	F5

10. Discard cuvettes: A1, B1, C1, D1, E1, F1.

At this point concentrations in cuvettes are as follows:

	2	3	4	5
A	00.000	00.000	00.000	00.000
B	59.423	59.423	59.423	59.423
C	69.327	59.327	59.327	59.327
D	79.231	79.231	79.231	79.231
E	89.135	89.135	89.135	89.135
F	99.039	99.039	99.039	99.039

The above concentrations assume a 100% initial concentration of the sample. Final concentrations can be determined from initial concentrations less than 100% by the following equations (modified from Microbics, 1992).

vol. S1	=	sample volume cuvette A1, B1, C1, D1, E1 or F1.
vol. R1	=	reagent volume cuvette A1, B1, C1, D1, E1 or F1.
S%	=	initial sample concentration.
SR%	=	sample concentration after reagent addition to cuvettes A1, B1, C1, D1, E1 or F1.
%S2		sample concentration in cuvettes 2 through 5
vol. S2	=	sample volume in cuvettes 2 through 5 before SR addition
vol. SR	=	sample + reagent volume.
SR%	=	$\left(\frac{vol\ S1}{vol\ S1+vol\ R1}\right) \times S\%$
Final Concentration	=	$\frac{(\%SR \times vol\ SR) + (\%S2 \times vol\ S2)}{vol\ SR + vol\ S2}$

Example: S% = 50%, Final concentration for F2

$$SR\% = \left(\frac{1250}{1250+50}\right) \times 50\% = 48.077$$

$$\text{Final Concentration} = \frac{(48.077 \times 250) + (50\% \times 750)}{250 + 750} = 49.519\%$$

11. When 5-minute timer sounds, place cuvette A2 in the READ Well. Press the SET button. When ready light goes on read and record the 5-minute light levels of each cuvette.
12. When the 15,minute timer sounds. Repeat step 11.
13. When the 30-minute timer sounds. Repeat step 11.
    Note: The above protocol does not depend on the computer to prompt the operator for readings, and precision requires some practice in developing the skill of making transfers and readings at a measured rhythm.

Data Reduction

Data recorded from the above was entered by keyboard into MTX7 software. Dunnet's hypothesis testing procedure was utilized to determine the most probable concentration of sample which caused no observable reduction in light from the Microtox® reagent.

APPENDIX F

MICROTOX REAGENT LIGHT INHIBITION OF 20% WSF

MICROTOX® REAGENT LIGHT INHIBITION OF 20% WSF

TABLE XI

MICROTOX® REAGENT LIGHT INHIBITION OF 20% WSF

Sample	Gamma (Γ) Value of 20% WSF	Microtox® Reagent Light Inhibition
0-1-1-1	0.19	15.97
0-1-2-1	0.09	8.42
0-1-3-1	0.19	15.97
0-1-4-1	0.27	21.26
0-2-1-1	0.39	28.06
0-2-2-1	2.00	66.67
0-2-3-1	1.1	52.38
0-2-4-1	0.3	23.08
2-2-C-2	0.27	21.26
2-2-C-3	0.5	33.3
0-3-1-1	0.6	37.5
0-3-2-1	1.00	50
0-3-3-1	0.28	21.88
0-3-4-1	0.75	42.86
3-4-C-2	0.35	25.93
3-2-1-1	1.1	52.38
3-2-2-1	0.7	41.18
3-2-3-1	1.3	56.52
3-2-4-1	0.67	40.12
3-3-1-1	0.09	7.83
3-3-2-1	0.15	13.04
3-3-3-1	0.1	8.93
3-3-4-1	0.14	12.28
4-2-1-1	0.71	41.52
4-2-2-1	0.3	23.08
4-2-3-1	1.7	62.96
4-2-4-1	0.5	33.33
4-2-C-2	0.47	31.97
4-3-1-1	0.27	21.26
4-3-2-1	0.12	10.71

TABLE XI

MICROTOX® REAGENT LIGHT INHIBITION OF 20% WSF

Sample	Gamma (Γ) Value of 20% WSF	Microtox® Reagent Light Inhibition
4-3-3-1	0.13	11.5
4-3-4-1	0.29	22.48
4-3-C-2	0.29	22.48
4-3-C-3	0.18	15.25
5-1-1-1	0.14	12.28
5-1-2-1	0.2	16.67
5-1-3-1	0.16	13.79
5-1-4-1	0.19	15.97
5-1-C-2	0.21	17.36
5-1-C-3	0.15	13.04
5-2-1-1	0.41	29.08
5-2-2-1	0.34	25.37
5-2-3-1	0.56	35.9
5-2-4-1	1.3	56.52
5-2-C-2	0.15	13.04
5-2-C-3	0.18	15.25
5-3-1-1	0.13	11.5
5-3-2-1	0.13	11.5
5-3-3-1	0.1	9.09
5-3-4-1	0.08	7.41
5-3-C-2	0.05	4.58
5-3-C-3	0.17	14.53
6-1-1-1	0.05	4.76
6-1-2-1	0.05	4.31
6-1-3-1	0.06	5.21
6-1-4-1	0.04	3.66
6-1-C-2	0.12	10.71
6-1-C-3	0.06	6.02
6-2-1-1	0.49	32.89
6-2-2-1	0.78	43.82
6-2-3-1	1.00	50.00
6-2-4-1	0.85	45.95
6-2-C-2	0.4	28.57
6-2-C-3	1.2	54.55
6-3-2-1	0.06	5.6
6-3-3-1	1.2	54.55

TABLE XI

MICROTOX® REAGENT LIGHT INHIBITION OF 20% WSF

Sample	Gamma (Γ) Value of 20% WSF	Microtox® Reagent Light Inhibition
6-3-4-1	0.07	6.1
6-3-C-2	0.2	16.67
6-3-C-3	0.09	8.26
7-1-1-1	0.06	5.57
7-1-2-1	0.06	5.48
7-1-3-1		0
7-1-4-1	0.06	5.66
7-1-C-2	0.05	4.31
7-1-C-3	0.05	4.31
7-2-1-1	1.4	58.33
7-2-2-1	2.0	66.67
7-2-3-1	2.2	68.75
7-2-4-1	2.6	72.22
7-2-C-2	0.48	32.43
7-2-C-3	0.7	41.18
7-3-1-1	0.08	7.41
7-3-2-1	0.07	6.54
7-3-3-1	0.11	9.91
7-3-4-1	0.12	10.71
7-3-C-2	0.12	10.71
7-3-C-3	0.17	14.53
8-1-1-1	0.05	4.76
8-1-2-1	0.05	4.31
8-1-3-1	0.05	4.31
8-1-4-1	0.04	3.57
8-1-C-2	0.05	4.76
8-1-C-3	0.05	4.94
8-2-1-1	0.79	44.13
8-2-2-1	0.56	35.9
8-2-3-1	0.8	44.44
8-2-4-1	1.5	60.0
8-2-C-2	0.19	15.97
8-2-C-3	0.26	20.63
8-3-1-1	0.14	12.28
8-3-2-1	1.01	50.25
8-3-3-1	0.09	8.34

TABLE XI

MICROTOX® REAGENT LIGHT INHIBITION OF 20% WSF

Sample	Gamma (Γ) Value of 20% WSF	Microtox® Reagent Light Inhibition
8-3-4-1	0.2	16.67
8-3-C-2	0.06	5.21
8-3-C-3	0.05	4.58
9-1-1-1	0.1	9.09
9-1-2-1	0.02	1.86
9-1-3-1	0.02	2.34
9-1-4-1	0.03	2.91
9-1-C-2	0.03	2.82
9-1-C-3	0.03	2.72
9-2-1-1	0.39	28.06
9-2-2-1	0.85	45.95
9-2-3-1	0.79	44.13
9-2-4-1	0.98	7.24
9-2-C-2	0.21	17.36
9-2-C-3	0.28	21.88
9-3-1-1	0.12	10.71
9-3-2-1	0.11	9.91
9-3-3-1	0.04	3.66
9-3-4-1	0.1	9.09
9-3-C-2	0.06	5.66
9-3-C-3	0.09	7.83

APPENDIX G

NO OBSERVED EFFECTS CONCENTRATION

AND LARGE SOILD PHASE TEST EC50

NO OBSERVED EFFECTS CONCENTRATION
AND LARGE SOLID PHASE TEST EC50

TABLE XII

DAY 190 TPH IR, EC50, NOEC AND LSPT EC50

Sample	TPH IR PPM	EC50 %	NOEC %	LSPT EC50 %
9-1-1-1	59	594.97	99.04	2.01
9-1-2-1	16	69,670	99.04	3.5
9-1-3-1	23	3,145	99.04	1.93
9-1-4-1	42	19,644	99.04	2.00
9-1-C-2	25	51,563	99.04	2.31
9-1-C-3	17	11,934	99.04	1.98
9-2-1-1	10,824	34.73	0.35	0.3
9-2-2-1	20,868	21.83	0.27	0.15
9-2-3-1	19,392	26.85	0.23	0.15
9-2-4-1	15,404	27.05	0.23	0.15
9-2-C-2	7,395	81.18	4.33	0.34
9-2-C-3	7,677	82.97		0.19
9-3-1-1	2,294	218.84	29.71	0.65
9-3-2-1	1,937	184.52	29.71	0.61
9-3-3-1	2,781	98.26	34.66	0.34
9-3-4-1	2,126	155.56	39.62	0.48
9-3-C-2	183	350.78	79.32	1.88
9-3-C-3	105	852.65	99.04	2.13

APPENDIX H

TYPICAL GAS CORHOMATOGRAPHS TEST PLOTS 2 AND 3 DAY 14 TO DAY 190

TYPICAL GAS CHROMATOGRAPHS TEST PLOTS 2 AND 3
DAY 14 TO DAY 190

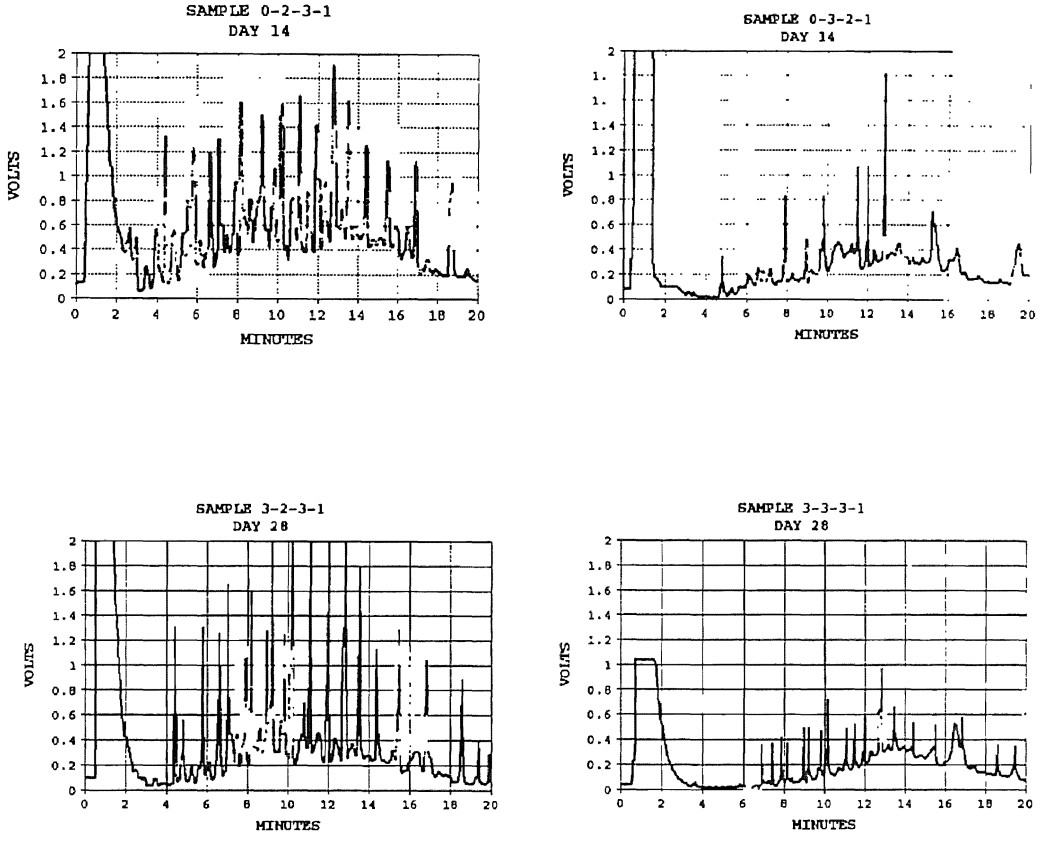


Figure 68. Typical Gas Chromatographs, Test Plots 2 and 3, Days 14 and 28

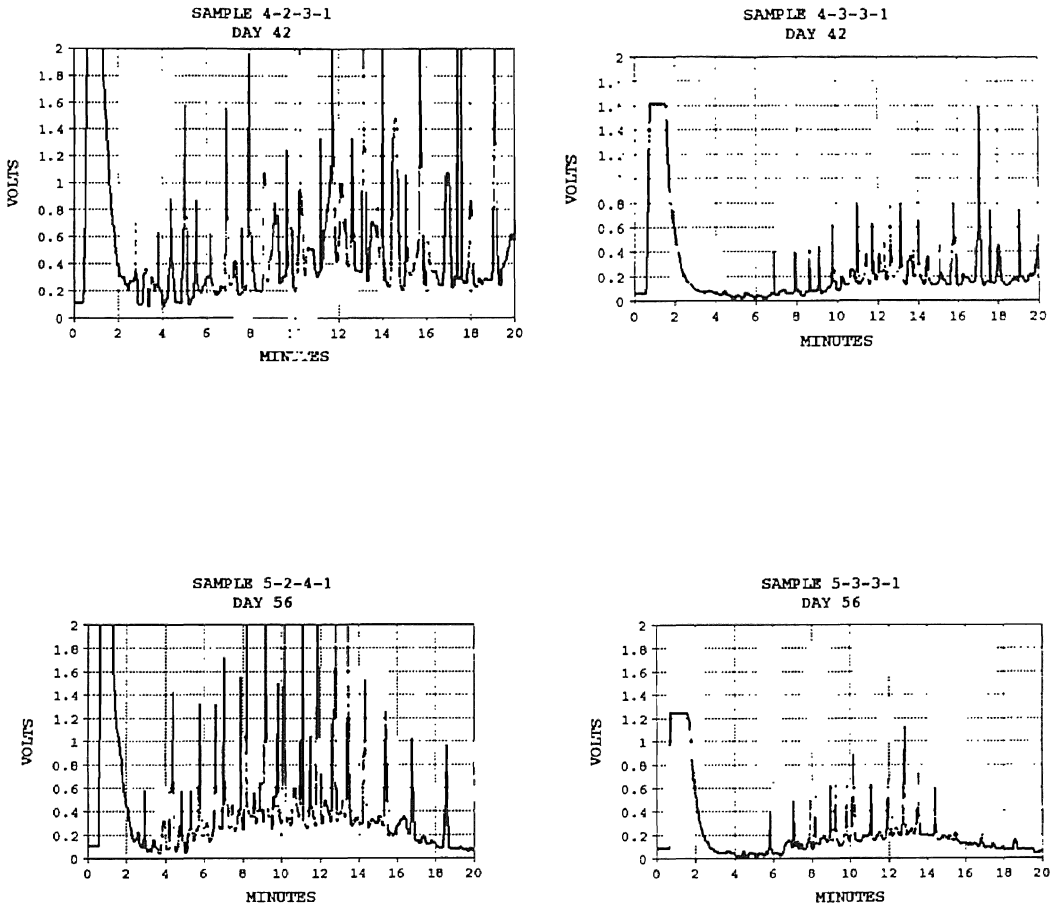


Figure 69. Typical Gas Chromatographs, Test Plots 2 and 3, Days 42 and 56

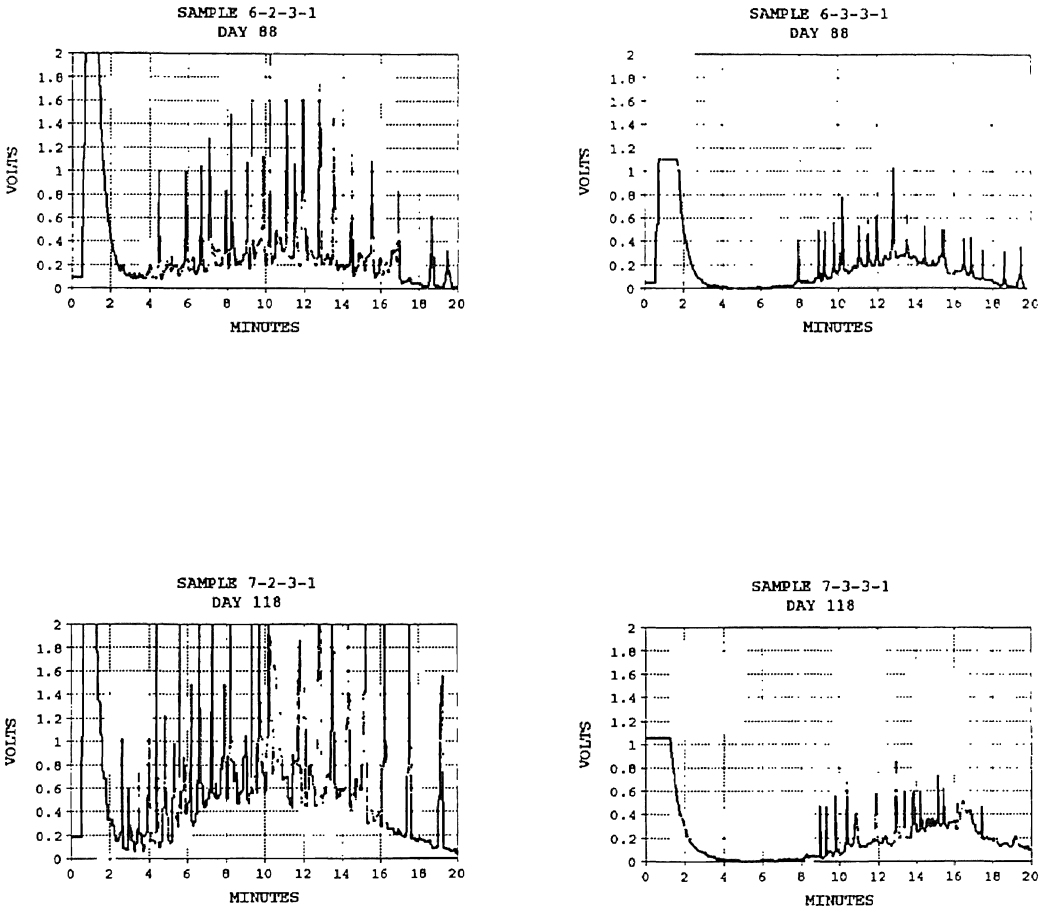


Figure 70. Typical Gas Chromatographs, Test Plots 2 and 3, Days 88 and 118

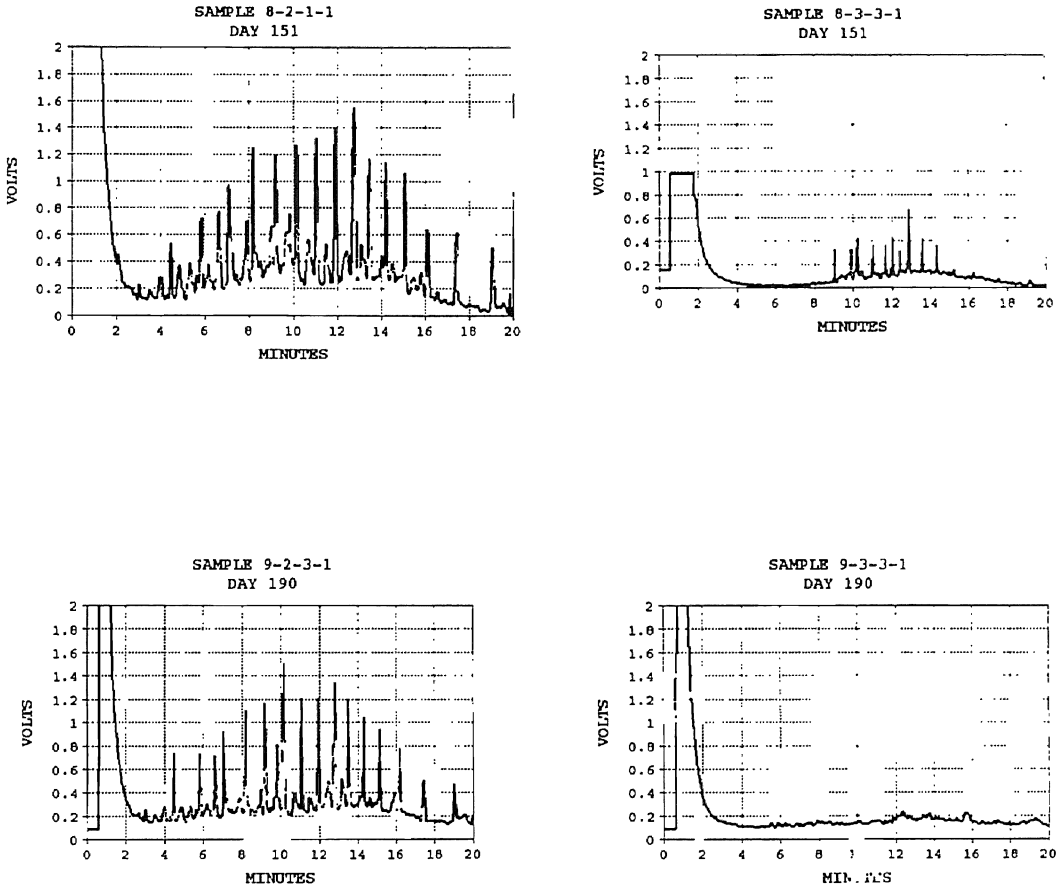


Figure 71. Typical Gas Chromatographs, Test Plots 2 and 3, Days151 and 190

VITA

Galen E. King, Jr.

Candidate for the Degree of

Master of Science

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